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(21) International Application Number: PCT/US91/01416 (22) International Filing Date: 1 March 1991 (01.03.91) (30) Priority data: 487,767 2 March 1990 (02.03.90) US (60) Parent Application or Grant (63) Related by Continuation US 487,767 (CIP) Filed on 2 March 1990 (02.03.90) (71) Applicant (for all designated States except US): BIO-TECH- NOLOGY GENERAL CORP. [US/US]; 1250 Broad- way, New York, NY 10001 (US).		(72) Invent rs; and (75) Inventors/Applicants (for US only) : GARFINKEL, Le- onard [US/US]; 16 Menuha Venahala Street 16/13, 00 230 Rehovot (IL). RICHTER, Tamar [IL/IL]; Habanim 115/23, 70 400 Nes Ziona (IL). (74) Agent: WHITE, John, P.; Cooper & Dunham, 30 Rocke- teller Plaza, New York, NY 10112 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), BR, CA, CH (European patent), DE (Eu- ropean patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European pa- tent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU, US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: CLONING AND PRODUCTION OF HUMAN VON WILLEBRAND FACTOR GPIb BINDING DOMAIN POLYPEPTIDES AND METHODS OF USING SAME (57) Abstract The subject invention provides non-glycosylated, biologically active polypeptides which comprise the vWF (von Wille- brand Factor) GPIb binding domain. These polypeptides may be used to inhibit platelet adhesion and aggregation in the treat- ment of subjects with conditions such as cerebrovascular disorders and cardiovascular disorders. This invention also provides ex- pression plasmids encoding these polypeptides as well as methods of producing by transforming a bacterial cell and recovering such polypeptides. In addition, the subject invention provides methods of treating and preventing cerebrovascular, cardiovascular and other disorders using these polypeptides to inhibit platelet aggregation.		

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CLONING AND PRODUCTION OF HUMAN VON WILLEBRAND FACTOR
GPIIb BINDING DOMAIN POLYPEPTIDES AND METHODS OF USING SAME

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Background of the Invention

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This application is a continuation-in-part of U.S. Serial No. 487,767, filed March 2, 1990, the contents of which are hereby incorporated by reference into the present disclosure.

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Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

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This invention relates to the cloning and production of human von Willebrand Factor analogs and methods of using such analogs.

Structural Features of von Willebrand Factor

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Von Willebrand Factor (vWF) is a large plasma protein which is synthesized in the endothelial cells which form the inner surface lining of the blood vessel wall, and by megakaryocytes, the precursor of platelets. Large amounts of vWF are found in platelet α -granules, whose contents are released into the blood upon platelet activation. Newly synthesized vWF in endothelial cells may enter the blood via two alternative pathways. Part is secreted constitutively into the blood, mainly as disulfide-linked dimers or small multimers of a 250,000 dalton subunit. Alternatively, part enters secretory organelles called Weibel-Palade bodies. The vWF stored in Weibel-Palade bodies is highly multimeric, ranging in size from that of a dimer to multimers of 50 or

more subunits, and can be released from the cells by treatment with secretagogues, such as thrombin. The highly multimeric vWF is the most effective in promoting platelet adhesion.

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The gene encoding vWF has been isolated and shown to be greater than 150 kb in length. It is composed of over 20 exons. The vWF mRNA is approximately 9000 bases in length and encodes a pre-pro-vWF of 2813 amino acids. Residues 1-
10 22 form a processed leader sequence which presumably is cleaved after entry of the protein into the rough endoplasmic reticulum. The N-terminal portion of the pro-vWF (741 amino acids) is the pro-peptide which is not present in mature vWF. This peptide is present in the blood
15 and has been shown to be identical to a blood protein previously known as von Willebrand Antigen II (vW AgII). The pro-peptide is essential for the multimerization of vWF. Cells into which a vWF cDNA containing only mature vWF sequences have been introduced produce only dimers. No
20 function is known for the propeptide/vW AgII.

DNA sequence analysis has demonstrated that the pro-vWF precursor is composed of repeated domain subunits. Four different domains have been identified. Mature vWF consists
25 of three A type, three B type, and two C type domains. There are also two complete and one partial D type domain. The pro-peptide consists of two D type domains, leading to the speculation that it may have associated functions.

30 Mature vWF is a multivalent molecule which has binding sites for several proteins. One of the binding sites recognizes the platelet glycoprotein Ib (GPIb). Using proteolytic digests this site has been localized to the region between amino acid residues 449 and 728 of mature vWF. In addition,
35 vWF has at least two collagen binding sites, at least two

heparin binding sites, a Factor VIII binding site, and a RGD site which binds to the platelet GP IIb/IIIa receptor.

Involvement Of vWF In Platelet Adhesion To Subendothelium

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Evidence that vWF, and specifically, the binding of vWF to the platelet GPIb receptor, is essential for normal platelet adhesion, is based on both clinical observations and in vitro studies. Patients with the bleeding disorder von Willebrand Disease (vWD) have reduced levels of vWF or are completely lacking in vWF. Alternatively, they may have defective vWF. Another disorder, Bernard-Soulier Syndrome (BSS), is characterized by platelets lacking GPIb receptors.

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15 The in vitro system which most closely approximates the environment of a damaged blood vessel consists of a perfusion chamber in which an everted blood vessel segment (rabbit aorta, human post-mortem renal artery, or the human umbilical artery) is exposed to flowing blood. After stripping off the layer of endothelial cells from the vessel, blood is allowed to flow through the chamber. The extent of platelet adhesion is estimated directly by morphometry or indirectly using radiolabeled platelets. Blood from patients with VWD or BSS does not support platelet adhesion in this system while normal blood does, indicating the need for vWF and platelet GPIb. Moreover, addition of monoclonal antibodies to GPIb prevents platelet adhesion as well. The vWF-dependence of platelet adhesion is more pronounced under conditions of high shear rates, such as that present in arterial flow. Under conditions of low shear rates, platelet adhesion may be facilitated by other adhesion proteins, such as fibronectin. Possibly, the adhesive forces provided by these other proteins are not adequate to support adhesion at high shear forces, and vWF dependence becomes apparent. Also, the multimeric nature of

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the vWF may provide for a stronger bond by binding more sites on the platelet.

5 About 20% of patients from whom clots have been removed by angioplasty or by administration of tissue plasminogen activator (tPA) suffer re-occlusion. This is presumably the result of damage to the endothelium during the treatment which results in the adhesion of platelets to the affected region on the inner surface of the vessel. This is followed
10 by the aggregation of many layers of platelets and fibrin onto the previously adhered platelets, forming a thrombus.

To date none of the anti-platelet aggregation agents described in the literature prevent the initial platelet
15 adhesion to the exposed sub-endothelium thereby preventing subsequent clot formation.

The subject invention provides non-glycosylated, biologically active polypeptides which comprise the vWF (von Willebrand Factor) GP1b binding domain. These polypeptides
20 may be used inter alia to inhibit platelet adhesion and aggregation in the treatment of subjects with conditions such as cerebrovascular disorders and cardiovascular disorders. This invention also provides expression plasmids
25 encoding these polypeptides as well as methods of producing by transforming a bacterial cell and recovering such polypeptides. In addition, the subject invention provides methods of treating and preventing cerebrovascular, cardiovascular and other disorders using these polypeptides
30 to inhibit platelet aggregation.

Summary of the Invention

This invention provides a non-glycosylated, biologically
5 active polypeptide having the amino acid sequence:

10 X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu Leu
Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
15 Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Phe
Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
20 Ile Val Ser Tyr Leu Cys]-B-COOH

wherein X is NH₂-methionine- or NH₂-;

25 A is a sequence of at least 1, but less than 35 amino
acids, which sequence is present in naturally occurring
vWF, the carboxy terminal amino acid of which is the
tyrosine #508 shown in Figure 12;

30 B is a sequence of at least 1, but less than 211 amino
acids, which sequence is present in naturally occurring
vWF, the amino terminal amino acid of which is the
aspartic acid #696 shown in Figure 12; and

the two cysteines included within the bracketed sequence
are joined by a disulfide bond.

In addition, the subject invention provides a method of producing any of the above-described polypeptides which comprises transforming a bacterial cell with an expression plasmid encoding the polypeptide, culturing the resulting bacterial cell so that the cell produces the polypeptide encoded by the plasmid, and recovering the polypeptide so produced.

Furthermore, the subject invention provides a pharmaceutical composition comprising an amount of any of the above-described polypeptides effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier. The subject invention also provides a method of inhibiting platelet aggregation which comprises contacting platelets with an amount of any of the above-described polypeptides effective to inhibit platelet aggregation. In addition, the subject invention provides methods of treating, preventing or inhibiting disorders such as cerebrovascular or cardiovascular disorders or thrombosis, comprising administering to the subject an amount of any of the above-described polypeptides effective to treat or prevent such disorders.

The subject invention also provides a method for recovering a purified, biologically active above-described polypeptide which comprises:

- (a) producing in a bacterial cell a first polypeptide having the amino acid sequence of the polypeptide but lacking the disulfide bond;
- (b) disrupting the bacterial cell so as to produce a lysate containing the first polypeptide;

- (c) treating the lysate so as to obtain inclusion bodies containing the first polypeptide;
- 5 (d) contacting the inclusion bodies from step (c) so as to obtain the first polypeptide in soluble form;
- 10 (e) treating the resulting first polypeptide so as to form the biologically active polypeptide;
- 15 (f) recovering the biologically active polypeptide so formed; and
- (g) purifying the biologically active polypeptide so recovered.

Brief Description of the FiguresFigure 1: Construction of pvW1P

5 This figure shows the construction of plasmid pvW1P. A series of vWF cDNA clones in λ gt11 (isolated from a human endothelial cell cDNA library) were isolated. One cDNA clone covering the entire GPIb binding domain was subcloned into the EcoRI site of pUC19. The resulting plasmid, pvW1P,
10 contains a 2.5 kb cDNA insert.

Figure 2: Construction of pvWF-VA1

15 This figure shows the construction of plasmid pvWF-VA1. A synthetic oligomer containing an ATG initiation codon located before the amino acid glu-437 (i.e., the 437th amino acid in the vWF protein shown in Figure 12) was ligated to plasmid pvW1P digested with NdeI and Bsu36I. The resulting plasmid was designated pvWF-VA1, and has been deposited in
20 E. coli strain S ϕ 930 under ATCC Accession No. 68530.

Figure 3: Construction of pvWF-VB1

25 This figure shows the construction of plasmid pvWF-VB1. A synthetic oligomer containing an ATG initiation codon located before the amino acid phe-443 (see Figure 12) was ligated to plasmid pvW1P digested with NdeI and Bsu36I. The resulting plasmid was designated pvWF-VB1.

30 Figure 4: Construction of pvWF-VA2

This figure shows the construction of plasmid pvWF-VA2. A synthetic oligomer containing a TAA termination codon located after the amino acid lys-728 (see Figure 12) was
35 ligated to plasmid pvWF-VA1 digested with HindIII and XmaI.

The resulting plasmid was designated pvWF-VA2.

Figure 5: Construction of pvWF-VB2

5 This figure shows the construction of plasmid pvWF-VB2. A synthetic oligomer containing a TAA termination codon was ligated to plasmid pvWF-VB1 digested with HindIII and XmaI. The resulting plasmid was designated pvWF-VB2.

10 **Figure 6: Construction of pvWF-VA3**

This figure shows the construction of plasmid pvWF-VA3. An NdeI-EcoRV fragment was isolated from plasmid pvWF-VA2 and ligated to plasmid pMF-945 (constructed as described in
15 Figure 11) digested with NdeI and PvuII. The plasmid obtained was designated pvWF-VA3. The plasmid expresses VA, a vWF GPIb binding domain polypeptide which includes amino acids 437 to 728 (see Figure 12) under the control of the deo P₁P₂ promoter.

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Figure 7: Construction of pvWF-VB3

This figure shows the construction of plasmid pvWF-VB3. An NdeI-EcoRV fragment was isolated from plasmid pvWF-VB2 and
25 ligated to plasmid pMF-945 digested with NdeI and PvuII. The plasmid obtained was designated pvWF-VB3. The plasmid expresses VB, a vWF GPIb binding domain polypeptide which includes amino acids 443 to 728 under the control of the deo P₁P₂ promoter.

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Figure 8: Construction of pvWF-VC3

This figure shows the construction of plasmid pvWF-VC3. A synthetic linker was ligated to pvWF-VA3 digested with NdeI
35 and Tth111I. The plasmid obtained was designated pvWF-VC3,

and has been deposited with the ATCC under ATCC Accession No. 68241. The plasmid expresses VC (also referred to as VCL or VC3), a vWF GPIb binding domain polypeptide which includes amino acids 504 to 728 (see Figure 12) under the control of the deo P₁P₂ promoter.

Figure 9: Construction of pvWF-VD3

This figure shows the construction of plasmid pvWF-VD3. A synthetic linker was ligated to pvWF-VA3 digested with NdeI and Tth111I. The plasmid obtained was designated pvWF-VD3. The plasmid expresses VD, a vWF GPIb binding domain polypeptide which includes amino acids 513 to 728 (see Figure 12) under the control of the deo P₁P₂ promoter.

Figure 10: Relative Alignment of Plasmids Expressing vWF-GPIb Binding Domain Polypeptides

This figure shows the relative alignment of the plasmids expressing the vWF-GPIb binding domain polypeptides. Also shown on the top two lines are representations of the vWF cDNA and the location of the GPIb binding domain coding region within the cDNA.

Figure 11: Construction of Plasmid pMF-945

This figure shows the construction of plasmid pMF-945. Plasmid pEFF-920 (in Escherichia coli SØ930, ATCC Accession No. 67706) was cleaved with BglII and NdeI, and the large fragment was isolated. This fragment was ligated to the small 540 bp fragment produced by cleaving plasmid pMF-5534 (ATCC Accession No. 67703) with BglII and NdeI. This produces plasmid pMF-945 which harbors the PAR sequence and in 5' and 3' order the deo P₁P₂ promoter sequences, the modified deo ribosomal binding site with an enhancer

sequence, a pGH analog coding sequence and the T₁T₂ transcription termination sequence. Plasmid pMF-945 is a high level expressor of pGH analog protein.

5 **Figure 12: Translated cDNA Sequence of Mature Human vWF**

This figure which consists of Figures 12A, 12B, 12C, 12D, 12E, 12F, 12G and 12H shows the translated cDNA sequence of mature human von Willebrand Factor.

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This sequence was compiled using the data disclosed by Verweij, C.L., et al., EMBO Journal 5: 1839-1847 (1986) and Sadler, J.E., et al., Proc. Natl. Acad. Sci. 82: 6394-6398 (1985). This nucleotide sequence commences with nucleotide number 2519 (where nucleotide 1 relates to the start of the coding sequence for the signal peptide) and terminates with nucleotide 8668, a total of 6150 nucleotides encoding mature vWF consisting of 2050 amino acids. The translated amino acid sequence commences with amino acid number 1 and terminates with amino acid number 2050. The corresponding nucleotide and amino acid designations are used throughout this application.

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25 **Figure 13: Translated Sequence of VC, the vWF GPIb Binding Domain Polypeptide Expressed by Plasmids pvWF-VC3 and pvWF-VCL**

This figure shows the translated sequence of the von Willebrand Factor GPIb binding domain polypeptide expressed by plasmids pvWF-VC3 (ATCC Accession No. 68241) and pvWF-VCL (ATCC Accession No. 68242).

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The first codon ATG encoding the translation initiation codon methionine has been added to the nucleotide sequence corresponding to nucleotides 4028 to 4702 of the sequence of

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Figure 12. This sequence encodes a polypeptide containing 225 amino acids (plus the initiation methionine) corresponding to amino acid Leu 504 to amino acid Lys 728 of Figure 12, i.e. 226 amino acids in total.

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Figure 14: Construction of pvWF-VCL

This figure shows the construction of plasmid pvWF-VCL. Plasmid pvWF-VC3 was digested with HindIII and StyI and the 860 base pair fragment isolated. This fragment was ligated with the large fragment isolated from the HindIII-StyI digest of plasmid pMLK-100. The resulting plasmid was designated pvWF-VCL and deposited in E.coli 4300(F⁻) with the ATCC under ATCC Accession No. 68242. This plasmid expresses VCL, the same vWF GPIb binding domain polypeptide as pvWF-VC3 (methionine plus amino acids 504-728), however under control of the λP_L promoter and the deo ribosomal binding site.

20 **Figure 15: Construction of Plasmid pvWF-VE2**

Plasmid pvWF-VA2 was digested with NdeI and PstI and the large fragment isolated. Synthetic oligomers No. 2 and No. 3 (shown in Figure 16) were treated with T4 polynucleotide kinase. The large pvWF-VA2 fragment was then ligated with synthetic oligomers No. 1 and No. 4, (shown in Figure 16) and with kinased oligomers No. 2 and No. 3. The resulting plasmid was designated pvWF-VE2.

30 **Figure 16: Synthetic Oligomers Used in Construction of pvWF-VE2.**

This figure shows the four synthetic linkers (Nos. 1-4) used in construction of pvWF-VE2.

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Figure 17: Construction of Plasmid pvWF-VE3

Plasmid pvWF-VE2 was digested with NdeI and HindIII and the small 770 bp fragment isolated and ligated with the large
5 fragment isolated from the NdeI-HindIII digest of plasmid pMLK-7891. The resulting plasmid was designated pvWF-VE3.

Figure 18: Construction of Plasmid pvWF-VEL

10 Plasmid pvWF-VE3 was digested with XmnI, treated with bacterial alkaline phosphatase (BAP), and further digested with NdeI and HindIII. Plasmid pMLK-100 was digested with NdeI and HindIII and treated with BAP. The two digests were
15 mixed and ligated, producing plasmid pvWF-VEL which expresses the DNA sequence corresponding to amino acids 469-728 of mature vWF under the control of the λP_L promoter and the cII ribosomal binding site.

Figure 19: The Effect of VCL on BJV-Induced Aggregation in Human Platelet Rich Plasma (PRP)

20 This figure provides the results of a standardized von Willebrand Factor (vWF)-dependent aggregation assay using human PRP.

Figure 20: The Effect of VCL on BJV-Induced Aggregation in Rat PRP

25 This figure provides the results of a standardized von Willebrand Factor (vWF)-dependent aggregation assay using rat PRP.

Detailed Description of the Invention

The plasmids pvWF-VC3, pvWF-VCL and pvWF-VA1 were deposited in Escherichia coli pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession Nos. 68241, 68242 and 68530, respectively.

This invention provides a non-glycosylated, biologically active polypeptide having the amino acid sequence:

15 X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu Leu
20 Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
25 Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Phe
Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
Ile Val Ser Tyr Leu Cys]-B-COOH

30 wherein X is NH₂-methionine- or NH₂-;

A is a sequence of at least 1, but less than 35 amino acids, which sequence is present in naturally occurring human vWF, the carboxy terminal amino acid of which is the tyrosine #508 shown in Figure 12;

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B is a sequence of at least 1, but less than 211 amino acids, which sequence is present in naturally occurring human vWF, the amino terminal amino acid of which is the aspartic acid #696 shown in Figure 12; and

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the two cysteines included within the bracketed sequence are joined by a disulfide bond. The bracketed sequence comprises amino acids #509-#695 of Figure 12.

10 In one embodiment, this polypeptide has the amino acid sequence:

X-[Leu His Asp Phe Tyr Cys Ser Arg Leu Leu Asp Leu Val
Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
15 Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr
His Asp Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys
Arg Pro Ser Glu Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr
Ala Gly Ser Gln Val Ala Ser Thr Ser Glu Val Leu Lys Tyr
Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu Ala
20 Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln
Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys
Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro
Glu Asn Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu
25 Gln Gln Arg Asp Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala
Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln
Val Thr Val Gly Pro Gly Leu Leu Gly Val Ser Thr Leu Gly
Pro Lys]-COOH

30 wherein X is NH₂- or NH₂-methionine-, preferably
NH₂-methionine-.

The bracketed sequence comprises amino acids #504-#728 of
Figure 12.

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One skilled in the art to which the subject invention pertains can readily make such polypeptides using recombinant or non-recombinant DNA techniques.

5 The polypeptides may be constructed using recombinant DNA technology. One means for obtaining the polypeptides is to express nucleic acid encoding the polypeptides in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known in the art, and recovering the
10 polypeptide after it has been expressed in such a host.

Examples of vectors that may be used to express the nucleic acid encoding the polypeptides are viruses such as bacteriophages (such as phage lambda), cosmids, plasmids,
15 and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. For example, using conventional restriction enzyme sites, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both
20 molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other
25 means are also available.

Vectors comprising nucleic acid encoding the polypeptides may be adapted for expression in a bacterial cell, a yeast cell, or a mammalian cell which additionally comprise the
30 regulatory elements necessary for expression of the nucleic acid in the bacterial, yeast, or mammalian cells so located relative to the nucleic acid encoding the polypeptide as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase
35 and transcription initiation sequences for ribosome binding.

For example, a bacterial expression vector may include a promoter such as the λ P_L or deo promoters and for transcription initiation the C_{II} or deo ribosomal binding sites. Such vectors may be obtained commercially or
5 assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

In addition, non-recombinant techniques such as chemical
10 synthesis, synthetic DNA or cDNA may be used to obtain the above-described polypeptides. One means of isolating the polypeptide is to probe a human genomic library with a natural or artificially designed DNA probe, using methods well known in the art. DNA and cDNA molecules which encode
15 the polypeptides may be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries.

20 The subject invention further provides a pharmaceutical composition comprising an amount of any of the above-described polypeptides effective to inhibit platelet aggregation and a pharmaceutically acceptable carrier.

25 As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers. Such carriers are well known in the art and may include, but are in no way and are not intended to be limited to, any of the standard pharmaceutical carriers such
30 as phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets, coated tablets, and capsules.

35 Typically such carriers contain excipients such as starch,

5 milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

10 The composition has an amount sufficient to result in a blood concentration of 0.06 to 58 μM , preferably between about 0.06 and 29 μM , for example 0.23 to 23 μM . Expressed in different terms, the amount should be 0.1 to 100 mg/Kg body weight, preferably 0.1 to 50 mg/Kg body weight, for example 0.4 to 40 mg/kg body weight.

15 The administration of the composition may be effected by any of the well known methods, including but not limited to intravenous, intramuscular, subcutaneous and oral administration.

20 This invention also provides a method of inhibiting platelet aggregation which comprises contacting platelets with an amount of any of the above-described polypeptides effective to inhibit platelet aggregation so as to inhibit platelet aggregation.

25 This invention also provides expression plasmids encoding the above-described polypeptides. In one embodiment, the expression plasmid encoding the polypeptide with the bracketed sequence, i.e. amino acids #504-#728 of Figure 12, is designated pvWF-VC3 and is deposited under ATCC Accession No. 68241. In another embodiment, the expression plasmid encoding a polypeptide with the bracketed sequence, i.e. amino acids #504-#728 of Figure 12, is designated pvWF-VCL and is deposited under ATCC Accession No. 68242.

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The expression plasmids of this invention further comprise suitable regulatory elements positioned within the plasmid relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell, such as promoter and operators, e.g. deo P₁P₂ and λ P_LO_L, ribosomal binding sites, e.g. deo and C_{II}, and repressors. Other suitable regulatory elements include, for example, the lac, trp, tac, and lpp promoters (European Patent Application Publication No. 0303972, published February 22, 1989).

The suitable regulatory elements are positioned within the plasmid relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell. In preferred embodiments of the invention, the regulatory elements are positioned close to and upstream of the DNA encoding the polypeptide.

The expression plasmids of this invention may be introduced into suitable host cells, preferably bacterial host cells. Preferred bacterial host cells are Escherichia coli cells. Examples of suitable Escherichia coli cells are strains SØ930 or 4300, but other Escherichia coli strains and other bacteria can also be used as host cells for the plasmids. Such bacteria include Pseudomonas aeruginosa and Bacillus subtilis.

The bacteria used as hosts may be any strain including auxotrophic (such as A1645), prototrophic (such as A4255), and lytic strains; F⁺ and F⁻ strains; strains harboring the cI⁸⁵⁷ repressor sequence of the λ prophage (such as A1645 and A4255); and strains deleted for the deo repressors and the deo gene (see European Patent Application Publication No. 0303972, published February 22, 1989). Escherichia coli strain A4255 (F⁺) has been deposited under ATCC Accession

No. 53468, and Escherichia coli strain A1645 has been deposited under ATCC Accession No. 67829.

5 The invention provides a bacterial cell which comprises these expression plasmids. In one embodiment, the bacterial cell is an Escherichia coli cell. In preferred embodiments, the invention provides an Escherichia coli cell containing the plasmid designated pvWF-VA1, deposited in E. coli strain S ϕ 930 with the ATCC under ATCC Accession No. 68530; pvWF-VA3; pvWF-VB3; pvWF-VC3, deposited in E. coli strain S ϕ 930
10 with the ATCC under ATCC Accession No. 68241; pvWF-VD3; or pvWF-VCL, deposited in E. coli strain 4300(F⁻) with the ATCC under ATCC Accession No. 68242.

15 All the E. coli host strains described above can be "cured" of the plasmids they harbor by methods well-known in the art, e.g. the ethidium bromide method described by R.P. Novick in Bacteriol. Review 33, 210 (1969).

20 In addition, the subject invention provides a method of producing any of the above-described polypeptides which comprises transforming a bacterial cell with an expression plasmid encoding the polypeptide, culturing the resulting bacterial cell so that the cell produces the polypeptide
25 encoded by the plasmid, and recovering the polypeptide so produced.

Furthermore, the invention provides a method of treating a subject with a cerebrovascular disorder which comprises
30 administering to the subject an amount of any of the polypeptides of the invention effective to inhibit platelet aggregation.

Also provided is a method of treating a subject with a
35 cardiovascular disorder which comprises administering to the

subject an amount of a polypeptide effective to inhibit platelet aggregation. Examples of cardiovascular disorders susceptible to treatment include acute myocardial infarction or angina.

5

Further, the subject invention provides method of inhibiting platelet aggregation in a subject prior to, during, or after the subject has undergone angioplasty, thrombolytic treatment, or coronary bypass surgery which comprises administering to the subject an amount of a polypeptide of the invention effective to inhibit platelet aggregation.

10

The invention also provides a method of maintaining blood vessel patency in a subject prior to, during, or after the subject has undergone coronary bypass surgery, which comprises administering to the subject an amount of a polypeptide of the invention effective to inhibit platelet aggregation.

15

The invention also provides a method of treating a subject having cancer which comprises administering to the subject an amount of a polypeptide of the invention effective to retard tumor metastasis.

20

The invention also provides a method of inhibiting thrombosis in a subject which comprises administering to the subject an amount of a polypeptide of the invention effective to inhibit the thrombosis. The thrombosis may be associated with an inflammatory response.

25

30

In addition, the subject invention provides a polypeptide of the invention bound to a solid matrix.

The invention also provides a method of treating a subject suffering from platelet adhesion to damaged vascular

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surfaces which comprises administering to the subject an amount of the polypeptide of the invention effective to inhibit platelet adhesion to damaged vascular surfaces.

5 The invention also provides a method of preventing platelet adhesion to a prosthetic material or device in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent platelet adhesion to the material or device.

10 The invention also provides a method of inhibiting re-occlusion in a subject following angioplasty or thrombolysis which comprises administering to the subject an amount of the polypeptide of the invention effective to inhibit re-occlusion.

15 The invention also provides a method of preventing vaso-occlusive crises in a subject suffering from sickle cell anemia which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent vaso-occlusive crises.

20 The invention also provides a method of preventing arteriosclerosis in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent arteriosclerosis.

25 The invention also provides a method of thrombolytic treatment of thrombi-containing, platelet-rich aggregates in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to treat thrombi-containing, platelet-rich aggregates.

30 The invention also provides a method of preventing platelet activation and thrombus formation due to high shear forces

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in a subject suffering from stenosed or partially obstructed arteries which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent platelet activation and thrombus formation.

5

The invention also provides a method of preventing thrombin-induced platelet activation in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent thrombin-induced platelet activation.

10

The invention also provides a method of preventing stenosis as a result of smooth muscle proliferation following vascular injury in a subject which comprises administering to the subject an amount of the polypeptide of the invention effective to prevent stenosis.

15

The invention also provides a method for recovering a purified, biologically active polypeptide of the invention which comprises:

20

(a) producing in a bacterial cell a first polypeptide having the amino acid sequence of the polypeptide but lacking the disulfide bond;

25

(b) disrupting the bacterial cell so as to produce a lysate containing the first polypeptide;

30

(c) treating the lysate so as to obtain inclusion bodies containing the first polypeptide;

35

(d) contacting the inclusion bodies from step

(c) so as to obtain the first polypeptide in soluble form;

5 (e) treating the resulting first polypeptide so as to form the biologically active polypeptide;

10 (f) recovering the biologically active polypeptide so formed; and

(g) purifying the biologically active polypeptide so recovered.

15 Step (e) may comprise contacting the polypeptide with a thiol-containing compound and disulfide so as to refold and reoxidize the polypeptide. Preferably, the thiol-containing compound is glutathione, thioredoxin, β -mercaptoethanol or cysteine.

20 The contacting of step (d) may be effected in the presence of a denaturant such as guanidine hydrochloride or urea.

The recovery of the polypeptide in step (f) may comprise removing the denaturant by dialysis.

25 In step (g), the biologically active polypeptide may be purified by cation exchange chromatography.

30 The first polypeptide may also be purified by cation exchange chromatography after step (d).

The examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be so construed as to, limit its scope in any way. The examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of genes encoding polypeptides of interest into such vectors or the introduction of the resulting plasmids into bacterial hosts. Such methods are well-known to those skilled in the art and are described in numerous publications including Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA, (1989).

EXAMPLES

All the references to map positions correspond to the identically numbered positions along the translated nucleotide sequence of mature human von Willebrand Factor shown in Figure 12.

Example 1: Cloning and Expression of vWF GPIb Binding Domain Polypeptides

cDNA Cloning of Human vWF GPIb Binding Domain

A human endothelial cDNA library (obtained from CLONTECH Laboratories, Inc.) in λ gt11 was screened for human vWF positive sequences using two synthetic DNA probes. The probes were synthesized according to the published DNA sequence (Sadler et al., Proc. Nat. Acad. Sci. 82: 6394-8 (1985) and Verweij et al., EMBO J. 3: 1829-47 (1986)) of human vWF (flanking 5' end and 3' end of the vWF domain known to bind the GPIb receptor) (see Figure 12).

The synthetic probes have the following sequences:

<u>Sequence</u>	<u>Nucleotides</u>
AAATCTGGCAGTGCTCAGGGTCACTGGGATTCAAGGTGAC	3863-3902
CCAGGACGAACGCCACATCCAGAACCATGGAGTTCCTCTT	4700-4739

5 A series of vWF cDNA clones covering the entire GPIb binding domain were identified and isolated. The cDNA fragments were subcloned into EcoRI site of pUC-19 (New England Biolabs, Inc.). One of the subclones, designated pvW1P (Figure 1), contains a 2.5 Kb insert. This 2.5 Kb insert
10 covers the entire GPIb binding domain extending from 550 bp upstream of the GPIb binding site to 1100 bp downstream of the GPIb binding site. (The subclone pvW1P has also been designated pvWF-1P).

15 Manipulation of DNA Coding for the vWF GPIb Binding Domain

In order to obtain expression of the GPIb binding domain in Escherichia coli under the regulation of the deo P₁P₂ promoter, the cDNA fragment of vWF, derived from plasmid
20 pvW1P was used for further manipulations as described below. As indicated previously, the vWF tryptic digest fragment that binds the GPIb receptor is from amino acid Val 449 to amino acid Lys 728.

25 A. Subcloning of the 5' end of vWF GPIb binding domain and addition of a translation initiation codon ATG.

Plasmid pvW1P has two convenient restriction sites at the 5' end. Bsu36I which cuts at the DNA sequence corresponding to
30 amino acid Ser (445), and Tth111I which cuts at amino acid Asp (514). Synthetic fragments of various size were designed that insert an ATG translation initiation codon at the 5' end as well as additional amino acids. This was done first, in order to maximize the chances of obtaining high
35 levels of expression. Second, they are a first step towards

reducing the size of the vWF GPIb binding domain peptide down to the minimal size needed, possibly eliminating collagen and heparin binding sites which may ultimately interfere with the function of the product.

5

A1. Amino acid Glu 437 at 5' end

Synthetic oligomers with the sequences:

10

5' - TATGGAGGTGGCTGGCCGGCGTTTTGCC - 3'

3' - ACCTCCACCGACCGCCGCAAAACGGAGT - 5'

NdeI

Bsu36I

15

were ligated to plasmid pvWF-1P digested with NdeI and Bsu36I (see Figure 2). The plasmid obtained was designated pvWF-VA1. Plasmid pvWF-VA1 has been maintained in E. coli strain SØ930 and was deposited under ATCC Accession No. 68530.

20

A2. Amino acid Phe 443 at 5' end

Synthetic oligomers with the sequences:

25

5' - TATGTTTTGCC - 3'

3' - ACAAACGGAGT - 5'

were ligated to plasmid pvW1P digested with NdeI and Bsu36I (see Figure 3). The plasmid obtained was designated pvWF-VB1.

30

B. Subcloning of the 3' end of vWF GPIb binding domain,
introduction of translation stop codon.

5 B1. Introduction of stop codon in plasmid pvWF-VA1

A synthetic oligomer with the sequence:

10 5'-CCGGGGCTCTTGGGGGTTTCGACCCTGGGGCCCAAGTAAAGATATCA-3'
3'-CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA-5'

15 was ligated to an XmaI and HindIII digested plasmid
pvWF-VA1 (see Figure 4). The plasmid obtained was
designated pvWF-VA2. This newly constructed
plasmid contains a translation termination codon
TAA adjacent to amino acid 728 (Lys) and EcoRV
site.

20 B2. Introduction of translation stop codon in plasmid
pvWF-VB1

A synthetic oligomer with the sequence:

25 5'- CCGGGGCTCTTGGGGGTTTCGACCCTGGGGCCCAAGTAAAGATATCA - 3'
3' - CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'

was ligated to plasmid pvWF-VB1 digested with XmaI and
HindIII. The plasmid obtained was designated pvWF-VB2
(see Figure 5).

30

Expression of the vWF GPIb binding domain in Escherichia
coli

35 In order to obtain expression of the vWF GPIb binding domain
various expression plasmids were constructed based on a deo

P₁P₂ constitutive promoter system.

1. Expression of a vWF GPIb binding domain polypeptide including amino acid Glu 437 to amino acid Lys 728 (based on plasmid pvWF-VA2)

An NdeI-EcoRV fragment was isolated from plasmid pvWF-VA2 and ligated into plasmid pMF-945 (see Figure 11) digested with NdeI and PvuII (see Figure 6). The plasmid obtained was designated as pvWF-VA3 and was maintained in Escherichia coli strain SØ930.

2. Expression of a vWF GPIb binding domain polypeptide including amino acid Phe 443 to amino acid Lys 728 (based on plasmid pvWF-VB2)

An NdeI-EcoRV fragment was isolated from plasmid pvWF-VB2 and ligated into plasmid pMF-945 digested with NdeI and PvuII (see Figure 7). The plasmid obtained was designated as pvWF-VB3 and was maintained in Escherichia coli strain SØ930.

3. Expression of a vWF GPIb binding domain polypeptide including amino acid Leu 504 to amino acid Lys 728 (based on expression plasmid pvWF-VA3)

A synthetic oligomer with the sequence:

5' - TATGTTGCACGATTTCTACTGCAGCAGGCTACTGGACC - 3'
3' - ACAACGTGCTAAAGATGACGTCGTCCGATGACCTGGA - 5'
NdeI Tth111I

was ligated to plasmid pvWF-VA3 digested with NdeI and Tth111I. The plasmid obtained was designated as pvWF-VC3 (see Figure 8). Plasmid pvWF-VC3 is maintained in Escherichia coli strain SØ930 and has been deposited with the ATCC under Accession No. 68241 (also see Figure 13).

4. Expression of a vWF GPIb binding domain polypeptide including amino acid Leu 513 to amino acid Lys 728 (based on expression plasmid pvWF-VA3)

A synthetic oligomer with the sequence:

5' - TATGCTGGACC - 3'
3'- ACGACCTGGA - 5'
NdeI Tth111I

was ligated to plasmid pvWF-VA3 digested with NdeI and Tth111I. The plasmid obtained was designated pvWF-VD3 (see Figure 9). Plasmid pvWF-VD3 is maintained in Escherichia coli strain SØ930.

Expression of vWF-GPIb binding domain polypeptides

The relative alignment of the expression plasmids is shown in Figure 10. Plasmids pvWF-VA3, pvWF-VB3, pvWF-VC3 and pvWF-VD3 in Escherichia coli strain SØ930 were used in order to analyze the levels of expression of the various vWF-GPIb binding domain peptides. The clones obtained were grown in LB medium containing Amp (100 µg/ml) at 37°C for 48 hours.

After 48 hours growth bacterial cells were harvested and centrifuged for 2 minutes at 10,000 RPM. Pellets were dissolved in 1/10 volume of 50 mM Tris-HCl pH=8.0. Sample

buffer (containing SDS and β -mercaptoethanol) was added. Samples were boiled for 10 minutes and loaded on a 10% SDS polyacrylamide gel. The expression of the vWF GPIb binding domain polypeptides in clones pvWF-VA3, pvWF-VB3 and pvWF-VD3 was low relative to the bacterial total proteins. The vWF polypeptides from these clones were detectable by Western blot analysis using commercially available polyclonal vWF antibody (Dekopatts a/s, Glostrup, Denmark). However, clones originated from Escherichia coli strain S ϕ 930 transformed with plasmid pvWF-VC3 expressed the vWF GPIb binding domain polypeptide (amino acid Leu 504 to amino acid Lys 728 plus methionine) at high levels (as a major band) detectable upon Coomassie staining.

Escherichia coli strain S ϕ 930 harboring plasmid pvWF-VC3 was deposited with the ATCC under Accession No. 68241. Subsequently, an inducible plasmid was constructed which contains the same vWF coding region as pvWF-VC3, expressed under the control of the λ P_L promoter and the deo ribosomal binding site (see Figure 14). This new plasmid, designated pvWF-VCL, proved to be a high expressor of VCL, the vWF GPIb binding domain polypeptide (methionine plus amino acid Leu 504 to amino acid Lys 728). This plasmid was deposited in Escherichia coli strain 4300 with the ATCC under Accession No. 68242. Escherichia coli strain 4300, constructed from Escherichia coli strain ATCC Accession No. 12435, is a wild-type, F⁻, biotin dependent strain, harboring the λ cI857 temperature-sensitive repressor. (A third plasmid construct harboring the same vWF coding region under the control of the λ promoter and the cII ribosomal binding site did not express any vWF peptide detectable by Coomassie staining.)

The NdeI-HindIII insert of pvWF-VCL can be conveniently subcloned into other expression vectors such as commercially

available pUC19 for production of a series of polypeptides which include the same amino acid sequence from amino acid 509 (cys) to amino acid 695 (cys) and have the same biological activity.

Example 2: Fermentation of Bacteria Expressing vWF GPIb Binding Domain Polypeptides

During scale-up fermentations of clone pvWF-VC3 it was found that the host tends to lose the plasmid due to instability. The loss of plasmids caused a reduction in vWF GPIb binding domain polypeptide expression. It was found necessary to maintain continuous selective pressure (i.e., continuous addition of Ampicillin) in order to maintain plasmid copy number and to maintain the expression levels. Large scale fermentation was carried out for 12 hours.

Fermentation was carried out in the following growth medium:

15	N-Z amino AS	20 gr
	Yeast extract	10 gr
	NaCl	5 gr
	K ₂ HPO ₄	2.5 gr
	MgSO ₄ ·7H ₂ O	1.0 gr
20	Anti foam	0.4 ml

Fructose (50%) was added to the growth medium at final concentration of 150 ml/liter and Ampicillin (100 mg/ml) was pumped continuously into the fermentor (total of 8 ml/liter). Fermentation was carried out for 12 hours at 37°C.

Purification of polypeptides

Cells were harvested after 12 hours fermentation and centrifuged. The bacterial pellet obtained was resuspended in buffer [50 mM Tris pH=8.0, 50 mM NaCl, 1 mM EDTA, 1mM DTT (dithiothreitol), 1 mM PMSF (Phenylmethylsulfonyl fluoride) and 10% Glycerol]. After additional centrifugation and sonication the vWF GPIb binding domain polypeptide was found

in the pellet.

The vWF GPIb binding domain polypeptide was further purified by solubilization of the pellet in 8M Urea containing 10 mM DTT, 25 mM Tris pH=8 and 1 mM EDTA at room temperature. The solubilized pellet was fractionated on a DEAE cellulose ion exchange column chromatography. (Elution buffer as above except 0.5 mM DTT).

10 The vWF GPIb binding domain polypeptide was eluted at 150 mM NaCl. After dilution to 50 mM NaCl (in the above buffer) the partially purified peptide was loaded on a Q-Sepharose column. Elution from the Q-Sepharose column was carried out at various NaCl concentrations (step elution). The vWF GPIb
15 binding domain peptide was pooled in four peaks which eluted at 100 mM, 200 mM, 250 mM and 500 mM NaCl. All four peaks were dialyzed against 150 mM NaCl and 50 mM Tris pH=8 for 36 hours. During the dialysis the Urea concentration of the dialysis solution was reduced in a linear gradient from 6M
20 Urea to no Urea.

Exempl 3: Biological Activity of vWF GPIb Binding Domain
Polypeptides

Platelet Aggregation Assays

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vWF preparation:

Human plasma-derived vWF was purified from human outdated blood bank plasma according to J. Loscalzo and R.I. Handin, Biochemistry 23: 3880-3886 (1984). The purified plasma-derived vWF was concentrated by Amicon 100,000 cut-off filter membrane, to a final concentration of 0.25 mg/ml.

Asialo-vWF preparation:

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The purified plasma-derived human vWF was desialyated according to L. DeMarco and S. Shapiro, J. Clin. Invest. 68: 321-328 (1981) with the following modifications:

- 20 1. The Neuraminidase used was from Vibrio cholera type II (Sigma).
2. The reaction mixture contained 0.2 Units enzyme/ mg protein and protease inhibitors according to the following concentrations: Benzamidine (20 mM),
25 Leupeptin (15 µg/ml) and Aprotinin 20 (U/ ml). The asialo-vWF was used for platelet aggregation without any further purification.

30 Platelet aggregation - Induced by Asialo-vWF

As stated above, soluble vWF does not bind to platelets via the GPIb receptor. Asialo-vWF, obtained by neuraminidase treatment to remove sialic acid residues, readily binds to
35 platelets via GPIb. Presumably, the desialation lowers the

net negative charge on the vWF, allowing it to bind to the negatively charged GPIb receptor. Asialo-vWF binding to platelets causes activation, release of ADP, and GP IIb/IIIa mediated aggregation. Platelet aggregation induced by asialo-vWF was carried out with 200 μ l of PRP (Platelet-rich plasma) (Fujimura Y., et al., J. Biol. Chem. 261: 381-385 (1986)) and 39 μ g/ml of asialo-vWF (final concentration) in a Lumi aggregometer. The results of inhibition of platelet aggregation with VC, the vWF GPIb binding domain polypeptide, are summarized in Table I.

VC (also referred to as VCL or VC3) is a vWF GPIb binding domain polypeptide which includes methionine plus amino acids 504-728 (see Figure 12).

15

vWF-Ristocetin induced platelet aggregation

Ristocetin-induced platelet aggregation in the presence of purified human intact vWF was carried out with washed human platelets according to Fujimura Y. et al., J. Biol. Chem. 261: 381-385 (1986).

20

The results of inhibition of platelet aggregation induced by ristocetin in the presence of intact vWF are summarized in Table II. Additional results using these assays are described in Example 5.

25

TABLE I

Inhibition of Asialo-vWF Induced Platelet Aggregation
(In PRP) by VC, a vWF GPIb Binding Domain Polypeptide

5

Q-Sepharose Fraction	VC concentration μ M	% Inhibition of Platelet Aggregation	
200 mM NaCl	6	76	64
250 mM NaCl	6	82	73
500 mM NaCl	6	89	79

10

TABLE II

Inhibition of Ristocetin Induced Platelet Aggregation
by VC, a vWF GPIb Binding Domain Polypeptide

Q-Sepharose Fraction	VC concentration in μM	% Inhibition of Platelet Aggregation
200 mM NaCl	10	76
	6	69
	3	38
	1	22
250 mM NaCl	10	86
	6	67
	3	44
	1	34
500 mM NaCl	10	100
	6	79
	3	68
	1	54
	0.25	38
Dialysis Buffer (control)	0	0

Exempl 4:**An Improved Method of Obtaining Pure, Oxidized, Folded and Biologically Active vWF GPIb Binding Domain Polypeptide**

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In Example 2, fermentation of cells harboring plasmid pvWF-VC3 was described. Subsequently, a preferred plasmid, pvWF-VCL was constructed as described in Example 1 and maintained in E.coli strain A4300. This host/plasmid system was
10 fermented essentially as known in the art for vectors containing a gene expressed under control of the λP_L promoter, see, for example coassigned EPO Patent Publication No. 173,280, published March 5, 1986, Example 5, pages 73-74 (without added biotin, thiamine, trace elements, and
15 ampicillin). In this improved method of purification of vWF GPIb binding domain polypeptide, a cell pellet of the above fermentation of A4300/pvWF-VCL was used.

In this improved method a purer and more active polypeptide
20 is produced than by the method disclosed in Example 2. The general scheme of the downstream process consists of steps A through H as follows:

A. Cell disruption and suspension of pellet: A pellet
25 containing the vWF GPIb binding domain polypeptide is obtained as described in Example 2, by sonication and centrifugation of a cell suspension in 50mM Tris-HCl pH=8, 50mM NaCl, 1mM EDTA, 1mM DTT, 1mM PMSF, and 10% Glycerol.

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The pellet containing the inclusion bodies is dissolved at about 10% w/v in a solution such that the final concentrations after dissolution are 8M urea, 20mM DTT, 20mM HEPES pH 8, and 100mM NaCl. The resulting solution
35 may be further purified by ion exchange chromatography as

described below. Alternatively, the inclusion bodies may be solubilized in a buffer containing 6M guanidine hydrochloride followed by buffer exchange to urea. The inclusion bodies may also be dissolved at different concentrations of urea, guanidine hydrochloride or any other denaturant or in the absence of denaturants, for example, at extremes of pH.

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B. Cation exchange chromatography: This step eliminates most of the contaminants and produces the vWF GPIb binding domain polypeptide at greater than 90% purity. Any cation exchange (e.g. carboxymethyl) method may be used in this step, but CM-Sepharose fast flow (Pharmacia) chromatography is preferred. The functional group may be carboxymethyl, a phospho group or sulphonic groups such as sulphopropyl. The matrix may be based on inorganic compounds, synthetic resins, polysaccharides, or organic polymers; possible matrices are agarose, cellulose, trisacryl, dextran, glass beads, oxirane acrylic beads, acrylamide, agarose/polyacrylamide copolymer (Ultrogel) or hydrophilic vinyl polymer (Fractogel). In a specific embodiment, the polypeptide is loaded onto a CM-Sepharose FF column equilibrated with 8M urea, 1mM DTT, 20mM HEPES pH 8, 100mM NaCl. Pure polypeptide elutes in 8M urea, 1mM DTT, 20mM HEPES pH 8 and 200mM NaCl. Up to about 30 OD₂₈₀ units of solubilized inclusion bodies may be loaded per ml CM-Sepharose FF. At this ratio the eluted polypeptide typically has a concentration of 4-5 OD₂₈₀/ml.

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C. Oxidation/Refolding: The polypeptide solution eluted from the cation exchange step above is treated with 6M guanidine hydrochloride (GuCl) to disrupt any aggregates. The polypeptide is then diluted to 0.05 OD₂₈₀/ml in 2M GuCl, pH 5-11, preferably 20mM HEPES pH 8, 0.1mM GSSG

(glutathione, oxidized form). This mixture is allowed to stand overnight at room temperature. The products are analyzed by gel filtration on fast protein liquid chromatography (FPLC) such as Superose 12 before proceeding. Analysis shows that this protein concentration reproducibly yields about 30% correctly oxidized monomers, and 70% S-linked dimers and multimers, as well as reduced and incorrectly oxidized monomers. A higher protein concentration gives a higher absolute yield of correctly oxidized monomers but a lower percentage yield due to increased formation of S-linked dimers and multimers. For example, a protein concentration of 0.1 OD₂₈₀/ml yields only 20% correctly oxidized monomers. Reducing the concentration to 0.025 OD₂₈₀/ml yields 35-40% correctly oxidized monomers but a lower absolute yield per liter oxidation. Oxidations may also be performed in urea instead of in GuCl, or in any other denaturant or in the absence of denaturants under appropriate buffer conditions in which, for example, pH, ionic strength, and hydrophobicity are varied. The preferred concentration of urea is in the range 0.5M to 10M, preferably 4M, and the preferred oxidant is GSSG in the range 0.01mM to 5mM preferably 0.1mM. Other oxidants such as CuCl₂ may be used or alternatively no oxidant may be added, thereby utilizing air oxidation only. For scale-up, 4M urea is the presently preferred solution for the oxidation step.

D. Concentration: The oxidation products are concentrated, preferably to about OD₂₈₀=1 by a tangential flow ultra-filtration system with a 30K cutoff membrane, such as a "MINITAN" or "PELLICON" system of Millipore. The filtrate is quite clear as the material is relatively clean and most of the contaminants are large enough not to pass through the 30K membrane. It is thus possible to

reuse the filtrate for performing oxidations. This results in considerable savings since large volumes of 2M GuCl are quite expensive. No difference in the oxidation products of oxidations performed in reused versus freshly prepared 2M GuCl was detectable by FPLC analysis.

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E. Dialysis: It is necessary to reduce the GuCl or urea concentration to less than 10mM. This is achieved by dialysis against 20mM HEPES pH8, 100mM NaCl. The dialysis was performed in dialysis tubing with 2-3 changes of buffer, but may be alternatively performed by diafiltration against the same buffer in a tangential flow ultrafiltration system with a 10K MW cutoff membrane.

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During dialysis, as the concentration of GuCl (or urea or other denaturant) decreases, a white precipitate forms. This precipitate contains about 80% of the protein yielded by step D comprising S-S linked dimers, reduced and incorrectly oxidized monomer and some contaminants which coeluted from the cation exchange step. The supernatant is nearly 100% correctly oxidized and refolded monomer at a concentration of 0.2 OD₂₈₀/ml, which is about 20% of the protein yield of step D. This selective precipitation of contaminants and undesirable forms of the protein as a result of dialysis was surprising and not predictable. The yield of correctly oxidized monomer can be greatly increased by recovery from the precipitate. This is done as follows: the solution is clarified by centrifugation. The supernatant is saved, and the pellet is treated with DTT to reduce S-S bonds and reoxidized as described above. The pellet is dissolved in a minimal volume of 6M GuCl, 20ml HEPES

pH 8, 150mM NaCl, 20mM DTT. The solution was passed through Sephadex G25 in a buffer similar to the dissolution buffer but containing only 1mM DTT (instead of 20mM). The eluate is then diluted to $OD_{280}=0.05$ and treated as in steps C, D and E above. This procedure may be repeated more than once as long as additional purified monomer is obtained. All of the supernatants are then combined.

10 F. Cation exchange: The combined supernatant of the dialysate of step E is concentrated by binding to CM Sepharose in 20mM HEPES pH8, 100mM NaCl. Elution is with 20mM HEPES pH8, 400mM NaCl. The eluate is exclusively monomeric despite the high salt concentration. 15 Concentrations of up to 3 mg/ml have been achieved by this method and that is not the upper limit. This step can alternatively be performed with Heparin-Sepharose which also binds the purified monomer in 10mM Tris pH 7.4, 150mM NaCl. Elution from Heparin-Sepharose is 20 performed using 10mM Tris-HCl pH 7.4, 500mM NaCl.

G. Dialysis: The product of the previous step is dialyzed against 20mM HEPES pH8, 150mM NaCl.

25 H. Storage: At this stage the purified vWF GPIb binding domain polypeptide may be lyophilized. Upon reconstitution in a volume of water equal to the volume before lyophilization, the resultant solution contains 30 exclusively monomeric protein showing no traces of dimers or other multimers on FPLC.

In a specific embodiment of this method the following 35 procedure was performed:

a) 10 gm inclusion bodies (comprising 0.43 g net dry weight) were dissolved in a final volume of 100ml 8M urea, 20mM DTT, 20mM HEPES pH 8, 100 mM NaCl.

5 b) The protein was loaded onto a CM Sepharose column equilibrated with 8M urea, 1mM DTT, 20mM HEPES pH 8, 100mM NaCl. The protein eluted at 200mM NaCl in 8M urea, 20mM HEPES pH 8, 1mM DTT, and was saved.

10 c) The saved eluate of the previous step was treated with 6M GuCl to eliminate any aggregates, and was then diluted to 0.05 OD₂₈₀/ml in 2M GuCl, 20mM HEPES pH 8, 0.1mM GSSG. Oxidation was performed overnight at room temperature. (Note that the oxidation step can be performed in the
15 presence of urea instead of GuCl.)

d) The oxidation products were concentrated to OD₂₈₀=1 by ultrafiltration on a "MINITAN" unit containing a 30K membrane.

20 e) The concentrate of the previous step was dialyzed with three buffer changes against 20mM HEPES pH 8, 100mM NaCl. During dialysis, as the GuCl concentration decreased, a white precipitate formed which was removed by centrifugation and reprocessed once as described above. The supernatants
25 were combined.

f) The combined supernatants were concentrated by binding to CM Sepharose in 20mM HEPES pH 8, 100mM NaCl. The polypeptide was eluted in 20mM HEPES, pH 8, 400mM NaCl and stored at 4°C.

35 g) The saved eluate from the previous step was dialyzed

against 20mM HEPES pH 8, 150mM NaCl at 4°C.

h) After dialysis, the purified vWF GPIb binding domain polypeptide, designated VCL, was lyophilized.

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Analysis of VCL

1. Amino acid sequence analysis of VCL purified as described above revealed that the N-terminal sequence is Met-Leu-His-Asp-Phe which is the expected sequence according to Figure 12 with the addition of an N-terminal methionine residue.

10

2. Examination of VCL on polyacrylamide gels revealed that VCL electrophoreses at lower apparent molecular weight under non-reducing conditions than under reducing conditions (beta-mercaptoethanol). This shift from compact to less compact configuration is consistent with the reduction of a disulfide bond. Such an intramolecular bond is formed between the cysteines at positions 509 and 695. (The shift in molecular weight is not large enough to be consistent with the reduction of an intermolecular bond.)

15

20

Example 5:Biological Activity of VCL, a vWF GPIb binding domain polypeptide

5

The vWF GPIb binding domain polypeptide produced as described in Example 4 was designated VCL and was assayed for biological activity as described below.

10 1. Ristocetin induced platelet aggregation (RIPA)

RIPA assay was performed as described in Example 3 in a reaction mix containing 2×10^8 platelets/ml, $1 \mu\text{g/ml}$ plasma vWF, and 1mg/ml ristocetin. A series of concentrations of VCL was tested and the IC_{50} of VCL in 3 assays was determined to be $0.2\text{--}0.3 \mu\text{M}$. 100% inhibition was achieved with about $1 \mu\text{M}$ VCL.

20 2. Asialo vWF induced platelet aggregation

Asialo vWF induced platelet aggregation assay was performed as described in Example 3 with $200 \mu\text{l}$ platelet-rich plasma (PRP) and $10 \mu\text{g/ml}$ asialo-vWF in a Lumi aggregometer. A series of concentrations of VCL was tested and the IC_{50} of VCL in this assay was determined to be $0.15 \mu\text{M}$, and complete inhibition by $0.5 \mu\text{M}$.

25 3. Effect of VCL on preformed aggregates

The effect of VCL on preformed aggregates made by RIPA was tested. Aggregates were formed as in paragraph (1) above in the absence of VCL. Addition of VCL to a concentration of $0.5 \mu\text{M}$ disrupted the aggregates instantaneously.

35

4. Inhibition of thrombin induced platelet aggregation

Thrombin induced platelet aggregation assay was performed using 0.025 unit/ml thrombin and stractan prepared platelets. A series of concentrations of VCL was tested and the IC_{50} of VCL in this assay was determined to be $0.3\mu M$. This is a surprising effect, since in a parallel experiment, VCL was not effective in inhibiting direct binding of [^{125}I] labelled thrombin to platelets.

5. Effect on platelet deposition under conditions of flow

In a model system consisting of an everted denuded human umbilical artery in a flow cell, platelet deposition may be determined. Whole human blood flows over the artery fragment. After 10-15 minutes, the flow is stopped and platelet deposition is determined microscopically. The IC_{50} of VCL in this system was determined to be about $1\mu M$.

All the above results are summarized in Table III.

The inhibitory activity of VCL on ristocetin-induced or asialo vWF-induced platelet aggregation, ristocetin-induced vWF binding, and platelet adhesion was lost upon reduction of the disulfide bond between the cysteines at positions 509 and 695. In some experiments, the reduced VCL precipitated out of solution.

TABLE III

Biological Activity of VCL,
a vWF GPIb Binding Domain Polypeptide.

5

Example 5 (Paragraph No.)	Assay	μM VCL
1	Ristocetin induced platelet aggregation	$\text{IC}_{50}=0.2-0.3$
2	Asialo vWF induced platelet aggregation	$\text{IC}_{50}=0.15$
3	Thrombin induced platelet aggregation	$\text{IC}_{50}=0.3$
4	Dissolution of preformed aggregates	0.5
5	Platelet deposition under conditions of flow	$\text{IC}_{50}=1$

10

Exempl 6:**Construction of Plasmid pvWF-VEL**

It was decided to construct a plasmid which expresses a slightly longer portion of the vWF GPIb binding domain than pvWF-VCL. The construction is shown in Figures 15-18 and described in the brief descriptions of the figures.

A. Construction of pvWF-VE2

Plasmid pvWF-VA2 (constructed as shown in Figure 4) was digested with NdeI and PstI and the large fragment isolated. Four synthetic oligomers shown in Figure 16 were prepared. Nos. 2 and 3 were treated with T4 polynucleotide kinase to add 5' phosphate. The above mentioned large fragment of pvWF-VA2 was ligated as shown in Figure 15 with the four oligomers (two kinased, and two non-kinased). The resulting plasmid shown in Figure 15 was designated pvWF-VE2.

B. Construction of plasmid pvWF-VE3

Plasmid pvWF-VE2 was digested with NdeI and HindIII and the 770 bp fragment containing the vWF GPIb binding domain was isolated. Plasmid pMLK-7891 was also digested with NdeI and HindIII and the large fragment was isolated. The resulting plasmid, shown in Figure 17, was designated pvWF-VE3.

C. Construction of plasmid pvWF-VEL

Plasmid pvWF-VE3 was digested with XmnI, dephosphorylated with bacterial alkaline phosphatase (BAP) and then digested with NdeI and HindIII. Plasmid pMLK-100 was digested with NdeI and HindIII and dephosphorylated with BAP. The two digests were then ligated to yield plasmid pvWF-VEL as shown in Figure 18. This plasmid expresses the DNA sequence

corresponding to amino acids 469-728 of mature vWF under the control of the λP_L promoter and the cII ribosomal binding site. The protein probably also includes an additional N-terminal methionine residue. A conservative base change was introduced into ala-473 changing GCC to GCA which also encodes alanine. This introduced an SphI site into the gene by changing GCCTGC to GCATGC.

Expression of pvWF-VEL in E. coli 4300(F⁻) yields a 29 kD protein which reacts strongly with a monoclonal anti-vWF antibody and will be referred to herein as VEL.

Exempl 7:Pharmaceutical Uses of vWF GPIb Binding Domain Polypeptide

5 Examples 1 and 4 describe the production and purification of a novel vWF GPIb binding domain polypeptide designated VCL. Some of the uses envisaged for VCL or for other vWF GPIb binding domain polypeptides are described below. Pharmaceutical compositions containing VCL or such other
10 polypeptides may be formulated with a suitable pharmaceutically acceptable carrier using methods and carriers well known in the art.

1. The VCL composition described above may be used for
15 prevention of platelet adhesion to damaged vascular surfaces (see Example 5, sub-section 5).

2. The VCL composition described above may be used for
20 disruption of platelet-rich aggregates (see Example 5, subsection 3).

3. The VCL composition described above may be used for
prevention of re-occlusion following angioplasty or
thrombolysis (see Bellinger et al., PNAS, USA, 84: 8100-8104
25 (1987), Prevention of occlusive coronary artery thrombosis by a murine monoclonal antibody to porcine von Willebrand Factor).

4. The VCL composition described above may be used for
30 prevention of platelet activation and thrombus formation due to high shear forces such as in stenosed or partially obstructed arteries or at arterial bifurcations (see Peterson et al., Blood 2: 625-628 (1987), Shear-induced
platelet aggregation requires von Willebrand Factor and
35 platelet membrane glycoproteins Ib and IIb-IIIa).

5. The VCL composition described above may be used for prevention of thrombosis and re-occlusion after angioplasty or thrombolysis due to thrombin activation of platelets (see Fuster et al., J. Am. Coll. Cardiol. 12: 78A-84A (1988),
5 Antithrombotic therapy after myocardial reperfusion in acute myocardial infarction).

6. The VCL composition described above may be used for prevention of platelet adhesion to and aggregation on
10 prosthetic materials (see Badimon et al., J. of Biomaterials Applications, 5: 27-48 (1990), Platelet interaction to prosthetic materials - role of von Willebrand Factor in Platelet Interaction to PTFE).

7. The VCL composition described above may be used for prevention of intramyocardial platelet aggregation in patients with unstable angina (see Davies et al.,
15 Circulation 73: 418-427 (1986), Intramyocardial platelet aggregation in patients with unstable angina suffering sudden ischemic cardiac death).

8. The VCL composition described above may be used for prevention of vasospasm and vasoconstriction following
25 arterial injury caused by angioplasty, thrombolysis or other causes (see Lam et al., Circulation 75: 243-248 (1987), Is vasospasm related to platelet deposition?) .

9. The VCL composition described above may be used for prevention of restenosis following angioplasty or
30 thrombolysis (see McBride et al., N. Eng. J. of Med. 318: 1734-1737 (1988), Restenosis after successful coronary angioplasty).

10. The VCL composition described above may be used for
35 prevention of vaso-occlusive crises in sickle-cell anemia

(see Wick et al., J. Clin. Invest. 80: 905-910 (1987), Unusually large von Willebrand multimers increase adhesion of sickle erythrocytes to human endothelial cells under controlled flow).

5

11. The VCL composition described above may be used for prevention of thrombosis associated with inflammatory response (see Esmon, Science 235: 1348-1352 (1987), The regulation of natural anticoagulant pathways).

10

12. The VCL composition described above may be used for prevention of arteriosclerosis (see Fuster et al., Circulation Res. 51: 587-593 (1982), Arteriosclerosis in normal and von Willebrand pigs).

15

13. The VCL composition described above may be used as an antimetastatic agent (see Kitagawa et al., Cancer Res. 49: 537-541 (1989), Involvement of platelet membrane glycoprotein Ib and IIb/IIIa complex in thrombin-dependent and -independent platelet aggregations induced by tumor cells).

20

Example 8

1. In Vitro Studies

5 For these studies VCL, or vehicle control, was made up fresh in sterile water (2.2 mg/ml stock).

A. Platelet Aggregation (PRP)

10 This is a standardized von Willebrand Factor (vWF)-dependent aggregation assay using human or rat platelet rich plasma (PRP). The addition of various concentrations of unfractionated Bothrops jararaca venom (BJV), which includes botrocetin and an additional thrombin-like component, or
15 ristocetin results in an aggregatory response in the absence of any additional agent. Using ristocetin (1.5 mg/ml) as the agonist 43 µg/ml VCL abolished the aggregation of human PRP. Ristocetin up to 5.0 mg/ml did not cause measurable aggregation of rat PRP. Using this assay system with BJV as
20 the agonist VCL at 83 µg/ml slightly inhibited the response of human PRP (Figure 19) but not rat PRP (Figure 20).

It is concluded that ristocetin is not a suitable agonist for inducing vWF dependent aggregation in the rat. Further,
25 it is not possible to monitor the effects of VCL ex vivo using BJV-induced aggregation of rat PRP. However, VCL does inhibit vWF-dependent aggregation in human PRP in vitro.

B. Platelet Thrombin Receptor Assay

30 This assay measures the inhibition of thrombin-induced platelet pro-coagulant expression and is briefly described below. Human washed platelets are incubated in a buffer which contains CaCl_2 , factor Xa, prothrombin, and human
35 alpha-thrombin for 60 min at 28°C. At the end of this

period an aliquot is transferred into a buffer containing S2238 and EDTA (to prevent any further thrombin generation). The S2238 reaction is terminated after 15 minutes at room temperature with acetic acid and the absorbance at 405 nm read. The amount of S2238 cleavage directly due to the added human alpha-thrombin is estimated by including a control which contains no prothrombin and this value is subtracted from all results. VCL was tested in this assay at a final concentration of 0.1 mg/ml.

This assay is sensitive to both thrombin inhibitors and thrombin receptor antagonists. In the presence of VCL the thrombin generation was 114% of control (n=2).

We therefore conclude that VCL is not a thrombin receptor antagonist in this system.

2. In Vivo Studies

Arterial Thrombosis Model (Rat)

This method is essentially a modification of the model of Shand et al., Thromb. Res. 45 505-515 (1987). The method we use routinely is outlined below.

Rats are labelled with ^{111}In platelets and ^{125}I fibrinogen. The dorsal aorta is clamped, using modified Spencer-Wells forceps, for 1 minute. After a 45 minute reperfusion period the damaged vessel is removed, washed in citrate and counted. Results are expressed as mg blood equivalents. Differences in radiolabel accumulation between placebo and drug-treated animals are calculated and expressed as a percentag inhibition.

For the purpose of the evaluation of VCL the route of

administration was by bolus intravenous injection. VCL was used at doses of 2mg/kg (n=5) and 4mg/kg (n=3). It was administered 1 minute prior to clamping. The vessel was then reperfused for twenty minutes. The antithrombotic effect was assessed at the 20 minute end point of the reperfusion. The shortening of the reperfusion time (as compared to routine) was designed to save compound. Appropriate vehicle controls (n=5 for both doses) were assessed.

10

It can be seen that under these conditions VCL inhibits thrombus formation in this model (Table IV). The inhibition is seen for the platelet (¹¹¹In) components of the thrombus at the 4mg/kg dose. The other changes do not reach statistical significance, thus VCL shows antithrombotic efficacy in this rat arterial model.

15

In conclusion, VCL exhibits an antithrombotic effect in this rat model of arterial thrombosis which may be dose dependent.

20

3. Discussion

From the present data it appears that the VCL interacts with the human platelet vWF receptor and hence inhibits platelet aggregation in human PRP. There is however a marked difference between species (rat vs. human) when comparing inhibition of platelet aggregation. The species specificity of this effect and the causal mechanism were not investigated further. At a practical level this meant we were unable to analyze ex vivo samples in order to correlate the effects of VCL on aggregation and arterial thrombosis. Hence the analysis and interpretation of the in vivo efficacy of VCL as an antithrombotic in the rat arterial thrombosis model is complicated by this factor.

35

Despite GPIb possessing binding sites for both vWF and thrombin it would appear that any effects of VCL on thrombin binding to GPIb do not translate into antagonism of thrombin-induced pro-coagulant expression.

5

Overall VCL shows an antithrombotic effect in the rat arterial thrombosis model. This inhibition may be due to its interference with the binding of vWF to its receptor.

TABLE IV

The Effect of VCL on Arterial Thrombus
Formation in the Rat Dorsal Aorta

% INHIBITION					
DOSE (mg/kg)	PLATELETS	P	FIBRINOGEN	P	N
4	61.3 ± 8.0	.01	34.7 ± 8.7	NS	3
2	25.54 ± 20.98	NS	22.78 ± 13.48	NS	5

The results are expressed as mean percentage inhibition ± standard error. The number of experiments in the treated groups are denoted in the table and in all cases were compared to a group of 5 control animals. Statistical analysis was performed on the raw data prior to transformation to percentage inhibition. NS = not statistically significant.

What is claimed is:

1. A non-glycosylated, biologically active polypeptide having the amino acid sequence:

5

10

15

20

X-A-[Cys Ser Arg Leu Leu Asp Leu Val Phe Leu Leu Asp Gly
Ser Ser Arg Leu Ser Glu Ala Glu Phe Glu Val Leu Lys Ala
Phe Val Val Asp Met Met Glu Arg Leu Arg Ile Ser Gln Lys
Trp Val Arg Val Ala Val Val Glu Tyr His Asp Gly Ser His
Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu L u
Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val
Ala Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile
Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Ala Leu
Leu Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn
Phe Val Arg Tyr Val Gln Gly Leu Lys Lys Lys Lys Val Ile
Val Ile Pro Val Gly Ile Gly Pro His Ala Asn Leu Lys Gln
Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn Lys Ala Ph
Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp Glu
Ile Val Ser Tyr Leu Cys]-B-COOH

wherein X is NH₂-methionine- or NH₂-;

25

A is a sequence of at least 1, but less than 35 amino acids, which sequence is present in naturally occurring human vWF, the carboxy terminal amino acid of which is the tyrosine #508 shown in Figure 12;

30

B is a sequence of at least 1, but less than 211 amino acids, which sequence is present in naturally occurring human vWF, the amino terminal amino acid of which is the aspartic acid #696 shown in Figure 12; and

35

the two cysteines included within the bracketed

sequence are joined by a disulfide bond.

2. A polypeptide of claim 1 having the amino acid sequence:

5
X-[Leu His Asp Phe Tyr Cys Ser Arg Leu Leu Asp Leu Val
Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr
10 His Asp Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys
Arg Pro Ser Glu Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr
Ala Gly Ser Gln Val Ala Ser Thr Ser Glu Val Leu Lys Tyr
Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp Arg Pro Glu Ala
Ser Arg Ile Ala Leu Leu Leu Met Ala Ser Gln Glu Pro Gln
15 Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly Leu Lys
Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro
Glu Asn Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu
Gln Gln Arg Asp Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala
20 Pro Glu Ala Pro Pro Pro Thr Leu Pro Pro Asp Met Ala Gln
Val Thr Val Gly Pro Gly Leu Leu Gly Val Ser Thr Leu Gly
Pro Lys]-COOH

wherein X is NH₂-methionine- or NH₂-.

- 25
3. A pharmaceutical composition comprising an amount
of a polypeptide of claim 1 or 2 effective to in-
hibit platelet aggregation and a pharmaceutically
acceptable carrier.
30
4. A method of inhibiting platelet aggregation which
comprises contacting platelets with an amount of a
polypeptide of claim 1 or 2 effective to inhibit
platelet aggregation so as to inhibit platelet
35 aggregation.

5. An expression plasmid encoding a polypeptide of claim 1.
- 5 6. An expression plasmid encoding a polypeptide of claim 2 designated pvWF-VC3 and deposited under ATCC Accession No. 68241.
- 10 7. An expression plasmid encoding a polypeptide of claim 2 designated pvWF-VCL deposited under ATCC Accession No. 68242.
8. A bacterial cell which comprises the expression plasmid of claim 5, 6, or 7.
- 15 9. An Escherichia coli cell of claim 8.
- 20 10. A method of producing a polypeptide of claim 1 which comprises transforming a bacterial cell with an expression plasmid encoding the polypeptide, culturing the resulting bacterial cell so that the cell produces the polypeptide encoded by the plasmid, and recovering the polypeptide so produced.
- 25 11. A method of producing a polypeptide of claim 2 which comprises transforming a bacterial cell with an expression plasmid encoding the polypeptide, culturing the resulting bacterial cell so that the cell produces the polypeptide encoded by the plasmid, and recovering the polypeptide so produced.
- 30 12. A method of treating a subject with a cerebrovascular disorder which comprises administering to the subject an amount of a polypeptide of claim 1 or 2 effective to inhibit platelet aggregation.

13. A method of treating a subject with a cardiovascular disorder which comprises administering to the subject an amount of a polypeptide of claim 1 or 2 effective to inhibit platelet aggregation.
- 5 14. A method of treating a subject in accordance with claim 13, wherein the cardiovascular disorder comprises acute myocardial infarction.
- 10 15. A method of treating a subject in accordance with claim 13, wherein the cardiovascular disorder comprises angina.
- 15 16. A method of inhibiting platelet aggregation in a subject prior to, during, or after the subject has undergone angioplasty, thrombolytic treatment, or coronary bypass surgery which comprises administering to the subject an amount of a polypeptide of claim 1 or 2 effective to inhibit platelet aggregation.
- 20 17. A method of maintaining blood vessel patency in a subject prior to, during, or after the subject has undergone coronary bypass surgery, which comprises administering to the subject an amount of a polypeptide of claim 1 or 2 effective to inhibit platelet aggregation.
- 25 18. A method of treating a subject having cancer which comprises administering to the subject an amount of a polypeptide of claim 1 or 2 effective to retard tumor metastasis.
- 30 19. A method of inhibiting thrombosis in a subject which comprises administering to the subject an
- 35

amount of a polypeptide of claim 1 or 2 effective to inhibit the thrombosis.

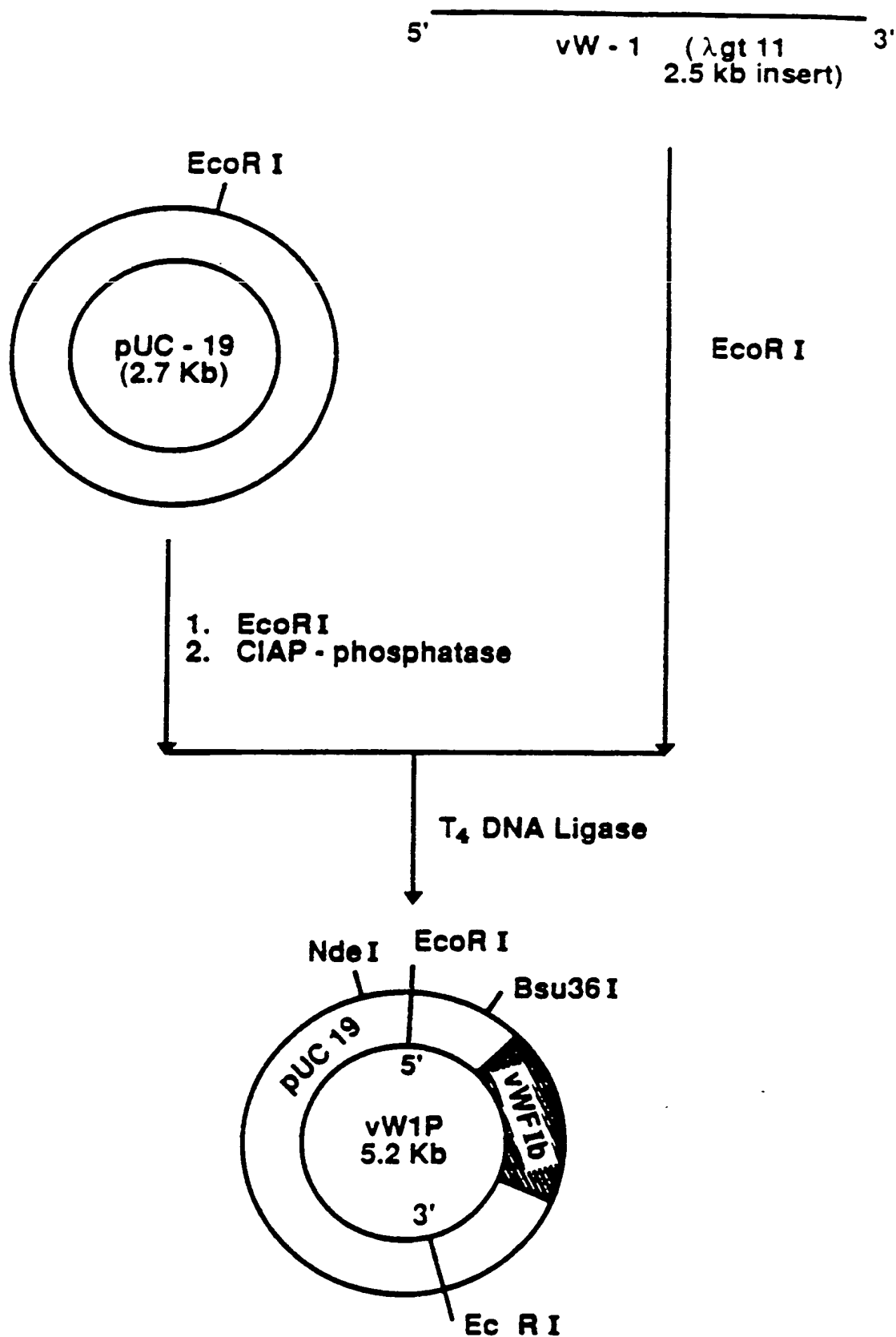
- 5 20. A polypeptide in accordance with claim 1 or 2 bound to a solid matrix.
- 10 21. A method of treating a subject suffering from platelet adhesion to damaged vascular surfaces which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to inhibit platelet adhesion to damaged vascular surfaces.
- 15 22. A method of preventing platelet adhesion to a prosthetic material or device in a subject which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent platelet adhesion to the material or device.
- 20 23. A method of inhibiting re-occlusion in a subject following angioplasty or thrombolysis which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to inhibit re-occlusion.
- 25 24. A method of preventing vaso-occlusive crises in a subject suffering from sickle cell anemia which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent vaso-occlusive crises.
- 30 25. A method of preventing arteriosclerosis in a subject which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent arteriosclerosis.
- 35

tive to pr vent arteriosclerosis.

- 5 26. A method of thrombolytic treatment of thrombi-containing, platelet-rich aggregates in a subject which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to treat thrombi-containing, platelet-rich aggregates.
- 10 27. A method of preventing platelet activation and thrombus formation due to high shear forces in a subject suffering from stenosed or partially obstructed arteries which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent platelet activation and thrombus formation.
- 15 28. A method of preventing thrombin-induced platelet activation in a subject which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent thrombin-induced platelet activation.
- 20 29. A method of preventing stenosis as a result of smooth muscle proliferation following vascular injury in a subject which comprises administering to the subject an amount of the polypeptide of claim 1 or 2 effective to prevent stenosis.
- 25 30. A method of claim 19, wherein the thrombosis is associated with an inflammatory response.
- 30 31. A method for recovering a purified, biologically active polypeptide of claim 1 which comprises:
- 35

- 5 (a) producing in a bacterial cell a first polypeptide having the amino acid sequence of the polypeptide but lacking the disulfide bond;
- (b) disrupting the bacterial cell so as to produce a lysate containing the first polypeptide;
- 10 (c) treating the lysate so as to obtain inclusion bodies containing the first polypeptide;
- 15 (d) contacting the inclusion bodies from step (c) so as to obtain the first polypeptide in soluble form;
- 20 (e) treating the resulting first polypeptide so as to form the biologically active polypeptide;
- (f) recovering the biologically active polypeptide so formed; and
- 25 (g) purifying the biologically active polypeptide so recovered.
32. A method of claim 31, wherein the treatment step (e) comprises contacting the polypeptide with a
- 30 thiol-containing compound and disulfide.
33. A method of claim 32, wherein the thiol-containing compound is glutathione, thioredoxin, β -mercaptoethanol or cysteine.

34. A method of claim 31, wherein the contacting of step (d) is effected in the presence of a denaturant.
- 5 35. A method of claim 34, wherein the denaturant is guanidine hydrochloride or urea.
- 10 36. A method of claim 34, wherein the recovery of the polypeptide of step (f) comprises removing the denaturant by dialysis.
- 15 37. A method of claim 31, wherein in step (g) the polypeptide is purified by cation exchange chromatography.
- 20 38. A method of claim 37, wherein the first polypeptide is purified by cation exchange chromatography after step (d).

1/35
Figure 1**SUBSTITUTE SHEET**

2/35

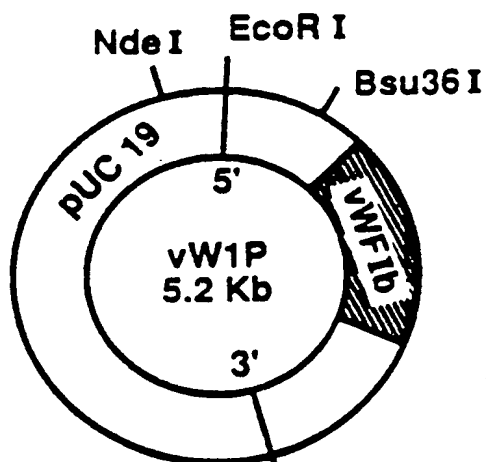
Figure 2

SYNTHETIC OLIGOMER

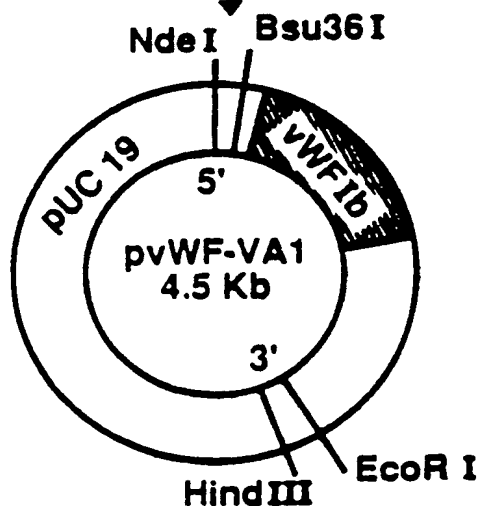
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 3' - ACTCCACCGACCGGCCGAAAACGGAGT - 5'

NdeI

BSU 36 I



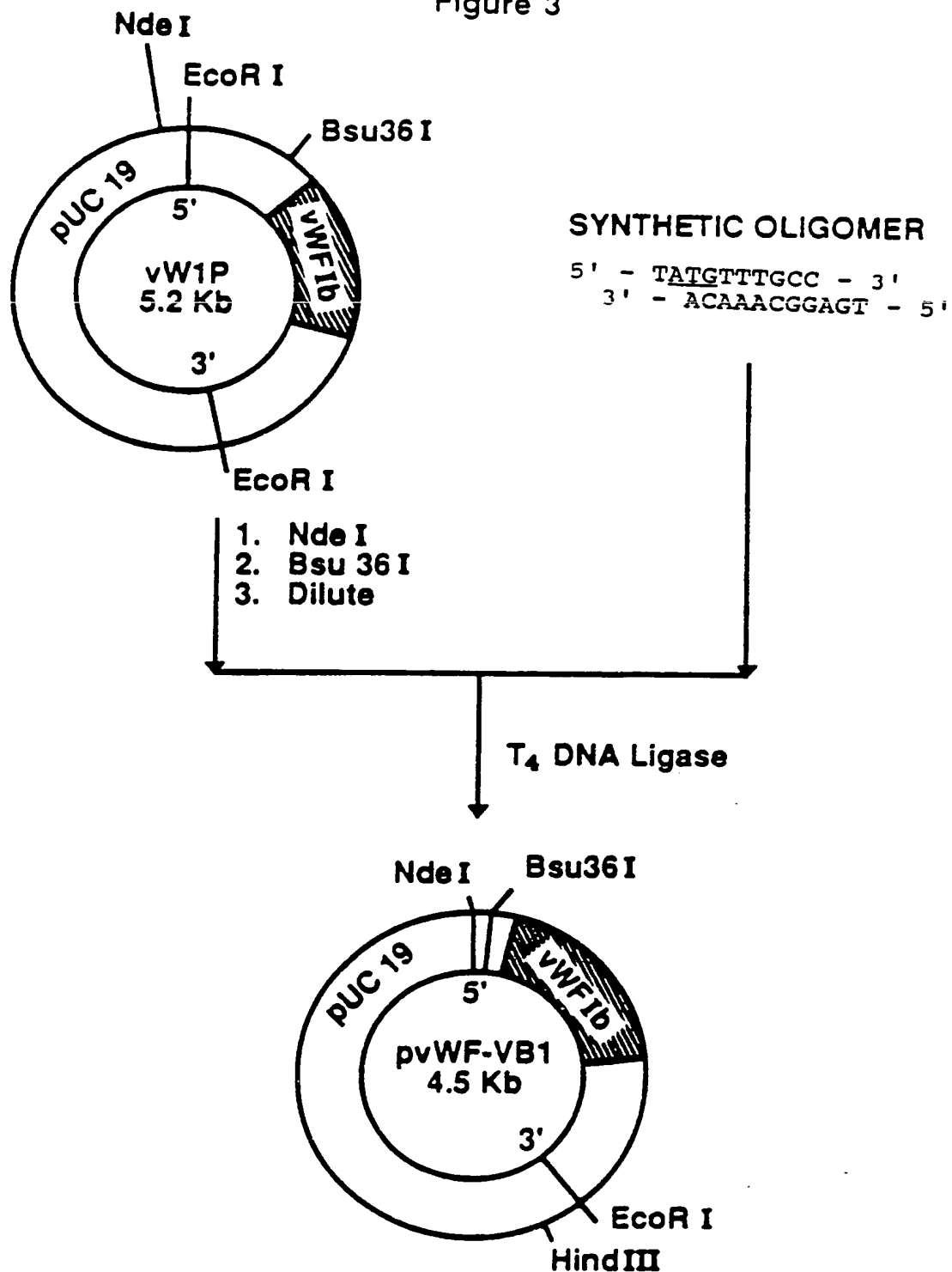
- EcoRI
1. Nde I
 2. Bsu 36 I
 3. Dilute

T₄ DNA Ligase

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Figure 3



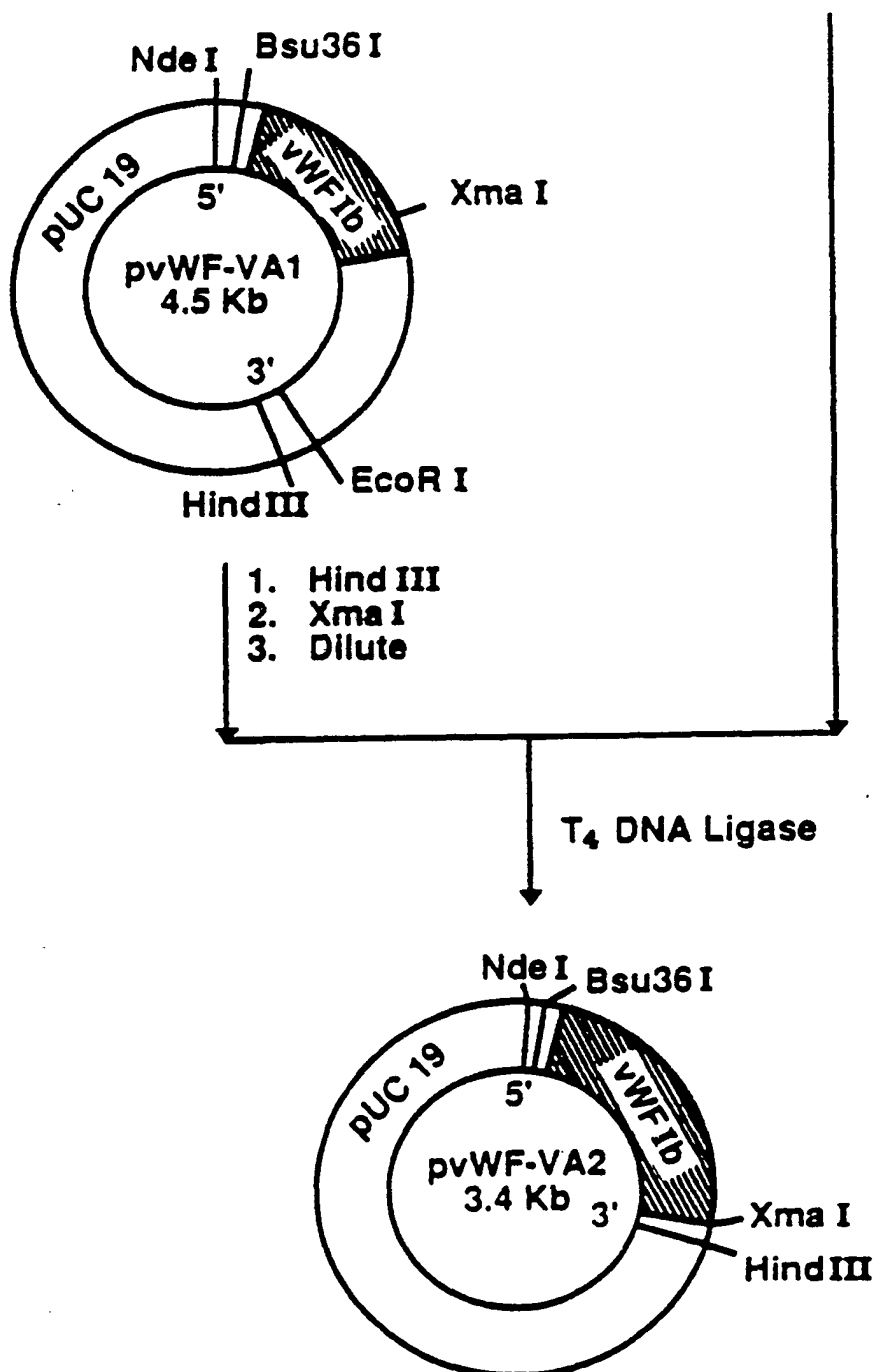
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Figure 4

SYNTHETIC LINKER

5' - CCGGGGCTCTTGGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA - 3'
 3' - CCGAGAACCCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'



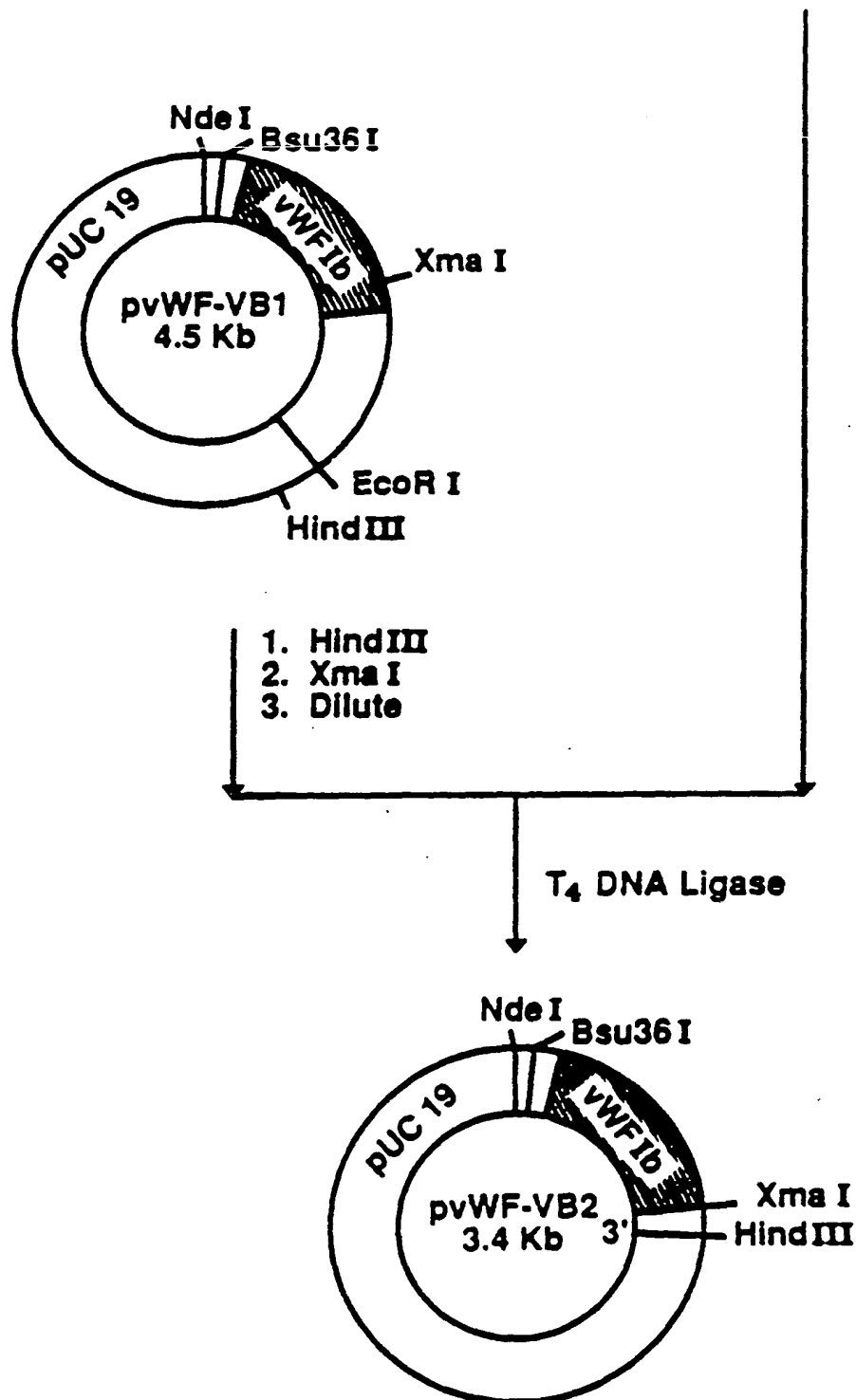
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5/35

Figure 5

SYNTHETIC LINKER

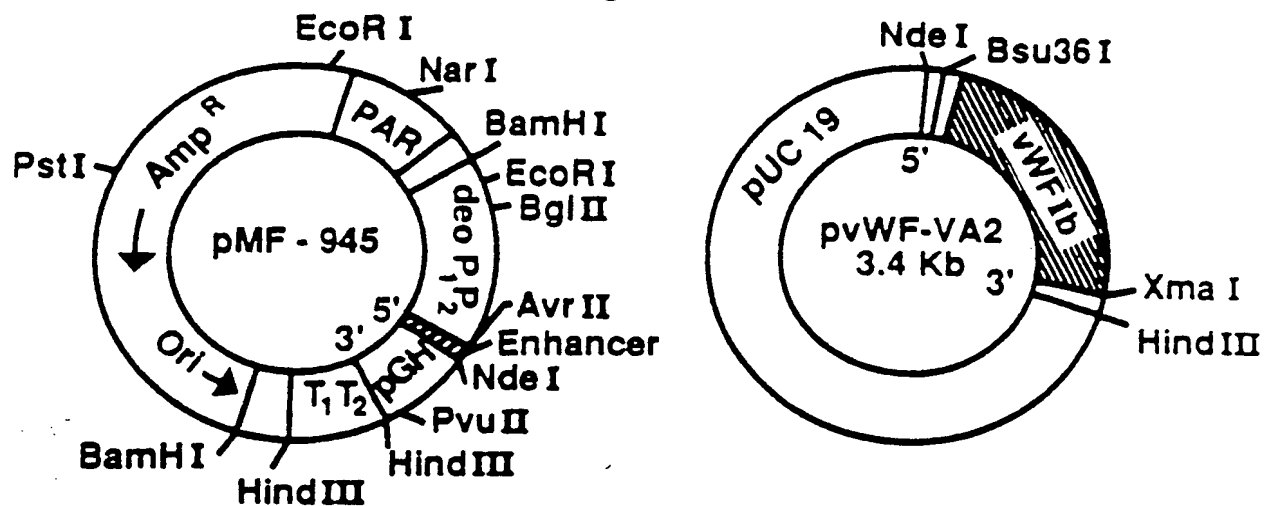
5' - CCGGGGCTCTTGGGGTTTCGACCCTGGGGCCCAAGTAAGATATCA - 3'
 3' - CCGAGAACCCCAAAGCTGGGACCCCGGGTTCATTCTATAGTTCGA - 5'



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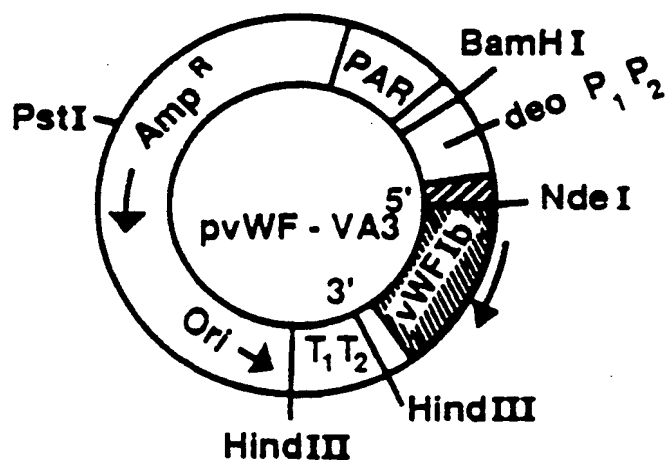
6/35

Figure 6



1. Nde I
2. Pvu II
3. Dilute

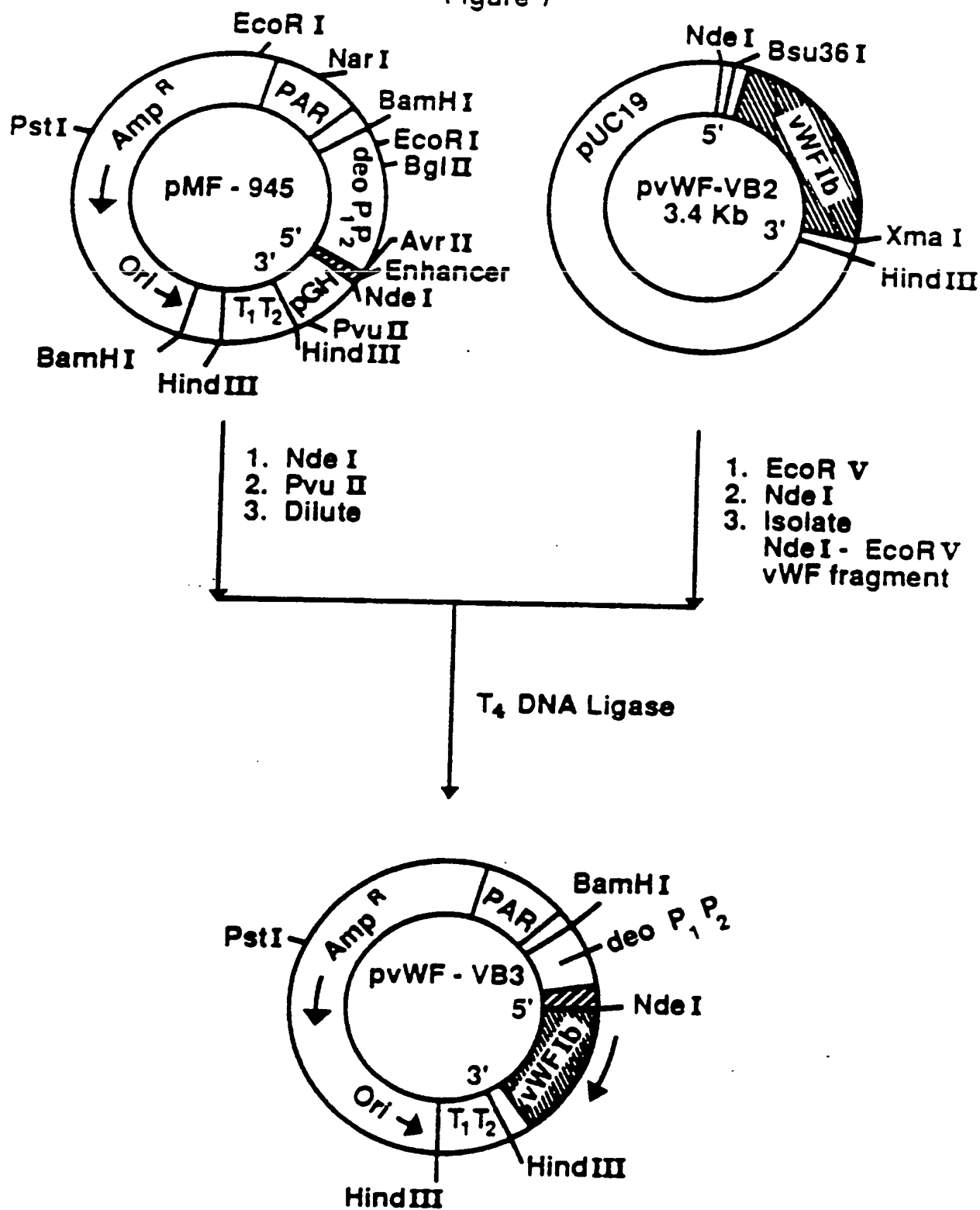
1. EcoR V
2. Nde I
3. Isolate
Nde I - EcoR V
vWF fragment

T₄ DNA Ligase

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Figure 7

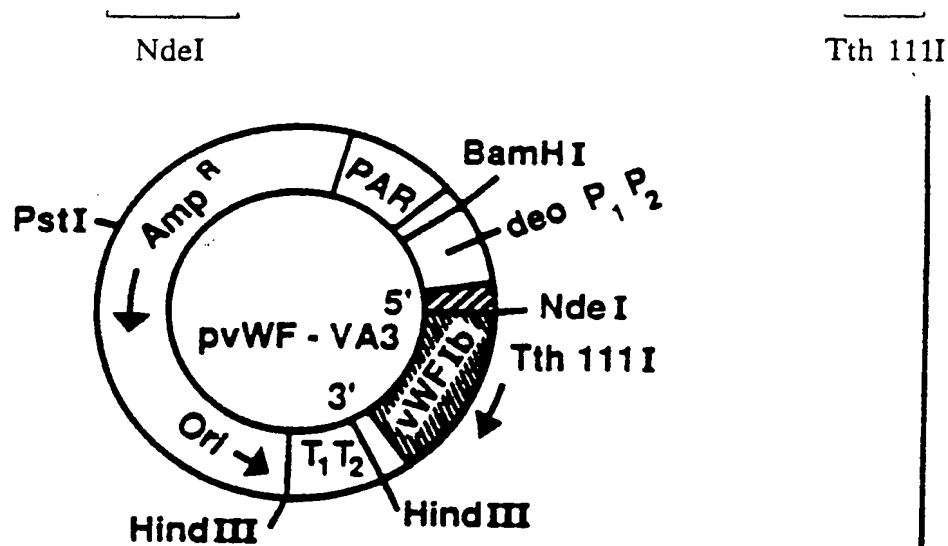


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8/35
Figure 8

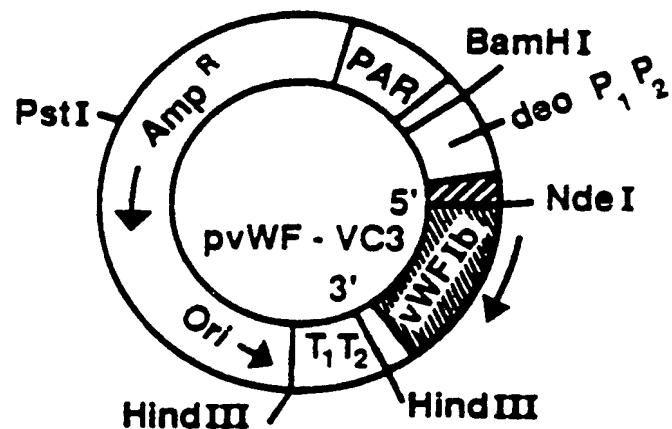
SYNTHETIC LINKER

5' - TATGTTGCACGATTTCTACTGCAGCAGGCTACTGGACC - 3'
3' - ACAACGTGCTAAAGATGACGTCGTCCGATGACCTGGA - 5'



1. Nde I
2. Tth 111 I
3. Dilute

T₄ DNA Ligase

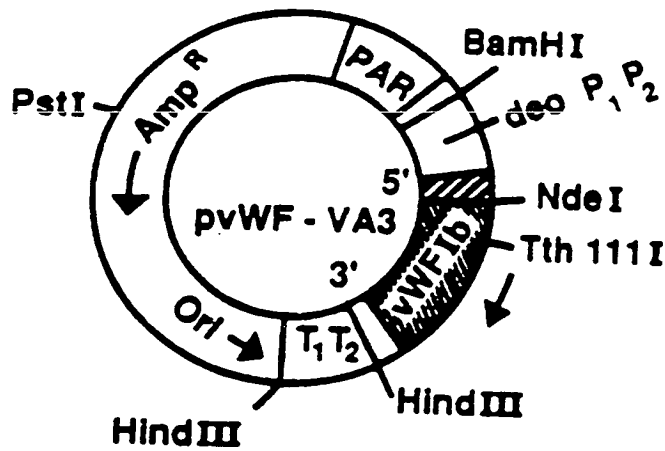
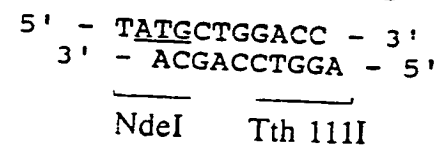


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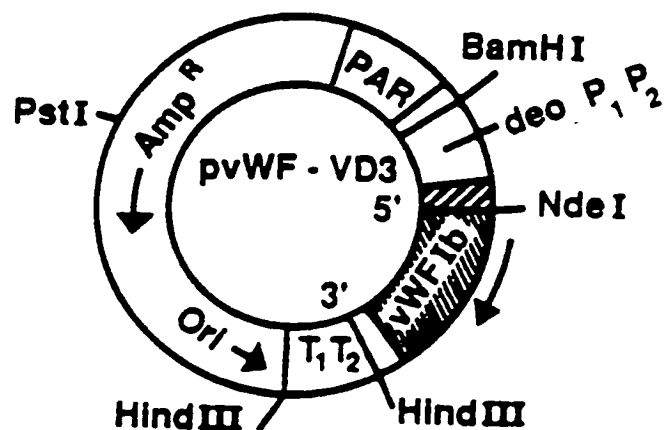
9/35

Figure 9

SYNTHETIC LINKERS



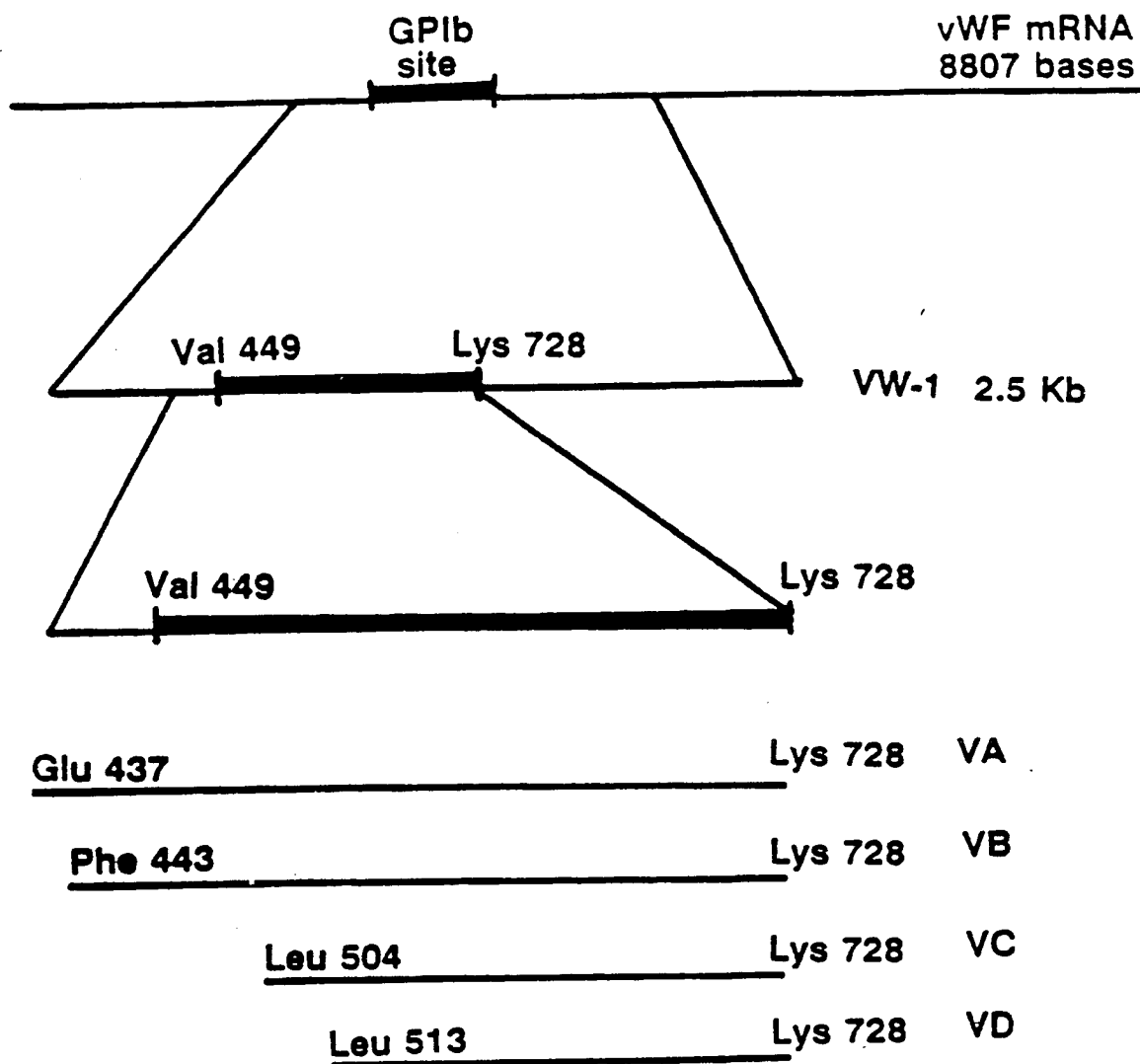
1. Nde I
2. Tth 111I
3. Dilute

T₄ DNA Ligase

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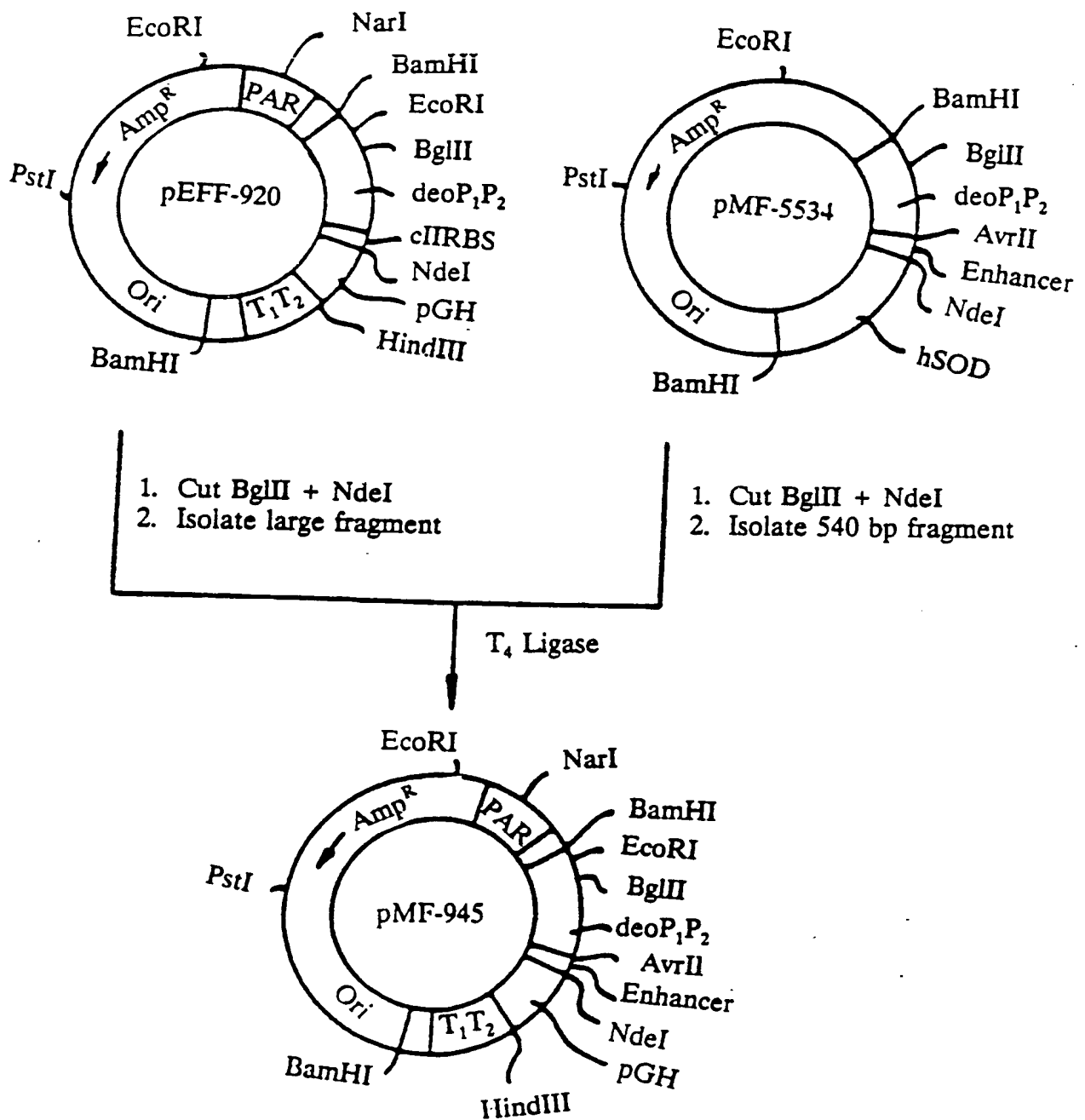
10/35

Figure 10



11/35

Figure 11



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Figure 12A

1	Ser	Leu	Ser	Cys	Arg	Pro	Pro	Met	Val	Lys	Leu	Val	Cys	Pro	Ala	Asp	16
2519	AGC	CTA	TCC	TGT	CGG	CCC	CCC	ATG	ATG	GTC	AAG	CTG	GTG	TGT	CCC	GCT	2566
17	Asn	Leu	Arg	Ala	Glu	Gly	Leu	Glu	Cys	Thr	Lys	Thr	Cys	Gln	Asn	Tyr	32
2567	AAC	CTG	CGG	GCT	GAA	GGG	CTC	GAG	TGT	ACC	AAA	ACG	TGC	CAG	AAC	TAT	2614
33	Asp	Leu	Glu	Cys	Met	Ser	Met	Gly	Cys	Val	Ser	Gly	Cys	Leu	Cys	Pro	48
2615	GAC	CTG	GAG	TGC	ATG	AGC	ATG	GGC	TGT	GTC	TCT	GGC	TGC	CTC	TGC	CCC	2662
49	Pro	Gly	Met	Val	Arg	His	Glu	Asn	Arg	Cys	Val	Ala	Leu	Glu	Arg	Cys	64
2663	CCG	GGC	ATG	GTC	CGG	CAT	GAG	AAC	AGA	TGT	GTG	CCC	CTG	GAA	AGG	TGT	2710
65	Pro	Cys	Phe	His	Gln	Gly	Lys	Glu	Tyr	Ala	Pro	Gly	Glu	Thr	Val	Lys	80
2711	CCC	TGC	TTC	CAT	CAG	GGC	AAG	GAG	TAT	GCC	CCT	GGA	GAA	ACA	GTG	AAG	2758
81	Ile	Gly	Cys	Asn	Thr	Cys	Val	Cys	Arg	Asp	Arg	Lys	Trp	Asn	Cys	Thr	96
2759	ATT	GGC	TGC	AAC	ACT	TGT	GTC	TGT	CGG	GAC	CGG	AAG	TGG	AAC	TGC	ACA	2806
97	Asp	His	Val	Cys	Asp	Ala	Thr	Cys	Ser	Thr	Ile	Gly	Met	Ala	His	Tyr	112
2807	GAC	CAT	GTG	TGT	GAT	GCC	ACG	TGC	TCC	ACG	ATC	GGC	ATG	GCC	CAC	TAC	2854
113	Leu	Thr	Phe	Asp	Gly	Leu	Lys	Tyr	Leu	Phe	Pro	Gly	Glu	Cys	Gln	Tyr	128
2855	CTC	ACC	TTC	GAC	GGG	CTC	AAA	TAC	CTG	TTC	CCC	GGG	GAG	TGC	CAG	TAC	2902
129	Val	Leu	Val	Gln	Asp	Tyr	Cys	Gly	Ser	Asn	Pro	Gly	Thr	Phe	Arg	Ile	144
2903	GTT	CTG	GTG	CAG	GAT	TAC	TGC	GGC	AGT	AAC	CCT	GGG	ACC	TTT	CGG	ATC	2950
145	Leu	Val	Gly	Asn	Lys	Gly	Cys	Ser	His	Pro	Ser	Val	Lys	Cys	Lys	Lys	160
2951	CTA	GTG	GGG	AAT	AAG	GGA	TGC	AGC	CAC	CCC	TCA	GTG	AAA	TGC	AAG	AAA	2998

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Figure 12A (continued)

161	Arg	Val	Thr	Ile	Leu	Val	Glu	Gly	Gly	Ile	Glu	Phe	Asp	Gly	176
2999	CGG	GTC	ACC	ATC	CTG	GAG	GGA	GGA	GAG	ATT	GAG	CTG	TTT	GAC	3046
177	Glu	Val	Asn	Val	Lys	Arg	Pro	Met	Lys	Asp	Glu	Thr	Phe	Glu	192
3047	GAG	GTC	AAT	GTC	AAG	AGG	CCC	ATG	AAG	GAT	GAG	ACT	CAC	TTT	3094
193	Val	Glu	Ser	Gly	Arg	Tyr	Ile	Ile	Leu	Leu	Gly	Lys	Ala	Leu	203
3095	GTC	GAG	TCT	GCG	CGG	TAC	ATC	ATT	CTG	CTG	CTG	GCG	AAA	GCC	3142
209	Val	Val	Trp	Asp	Arg	His	Leu	Ser	Ile	Ser	Val	Val	Leu	Lys	224
3143	GTC	GTC	TGG	GAC	CGC	CAC	CTG	AGC	ATC	TCC	CTG	CTC	AAG	CAG	3190
225	Tyr	Gln	Glu	Lys	Val	Cys	Gly	Leu	Cys	Gly	Asn	Phe	Asp	Gly	240
3191	TAC	CAG	GAG	AAA	GTC	TGT	GCG	CTG	TGT	GGG	AAT	TTT	GAT	GGC	3238
241	Asn	Asn	Asp	Leu	Thr	Ser	Ser	Asn	Leu	Gln	Val	Glu	Glu	Asp	256
3239	AAC	AAT	GAC	CTC	ACC	AGC	AGC	AAC	CTC	CAA	GTG	GAG	GAG	GAC	3286

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Figur 12B

257	Asp	Phe	Gly	Lys	Ser	Trp	Glu	Val	Ser	Ser	Gln	Cys	Ala	Asp	Thr	Arg	272
3287	GAC	TTT	GGG	AAG	TCC	TGG	GAA	GTG	ACC	TCG	CAG	TGT	GCT	GAC	ACC	AGA	3334
273	Lys	Val	Pro	Leu	Asp	Ser	Ser	Pro	Ala	Thr	Cys	His	Asn	Asn	Ile	Met	288
3335	AAA	GTG	CCT	CTG	GAC	TCA	TCC	CCT	GCC	ACC	TGC	CAT	AAC	AAC	ATC	ATG	3382
289	Lys	Gln	Thr	Met	Val	Asp	Ser	Ser	Cys	Arg	Ile	Leu	Thr	Ser	Asp	Val	304
3383	AAG	CAG	ACG	ATG	GTG	GAT	TCC	TCC	TGT	AGA	ATC	CTT	ACC	AGT	GAC	GTC	3430
305	Phe	Gln	Asp	Cys	Asn	Lys	Leu	Val	Asp	Pro	Glu	Pro	Tyr	Leu	Asp	Val	320
3431	TTC	CAG	GAC	TGC	AAC	AAG	CTG	GTG	GAC	CCC	GAG	CCA	TAT	CTG	GAT	GTC	3478
321	Cys	Ile	Tyr	Asp	Thr	Cys	Ser	Cys	Glu	Ser	Ile	Gly	Asp	Cys	Ala	Cys	336
3479	TGC	ATT	TAC	GAC	ACC	TGC	TCC	TGT	GAG	TCC	ATT	GGG	GAC	TGC	GCC	TGC	3526
337	Phe	Cys	Asp	Thr	Ile	Ala	Ala	Tyr	Ala	His	Val	Cys	Ala	Gln	His	Gly	352
3527	TTC	TGC	GAC	ACC	ATT	GCT	GCC	TAT	GCC	CAC	GTG	TGT	GCC	CAG	CAT	GGC	3574
353	Lys	Val	Val	Thr	Trp	Arg	Thr	Ala	Thr	Leu	Cys	Pro	Gln	Ser	Cys	Glu	368
3575	AAG	GTG	GTG	ACC	TGG	AGG	ACG	GCC	ACA	TTG	TGC	CCC	CAG	AGC	TGC	GAG	3622
369	Glu	Arg	Asn	Leu	Arg	Glu	Asn	Gly	Tyr	Glu	Cys	Glu	Trp	Arg	Tyr	Asn	384
3623	GAG	AGG	AAT	CTC	CGG	GAG	AAC	GGG	TAT	GAG	TGT	GAG	TGG	CGC	TAT	AAC	3670
385	Ser	Cys	Ala	Pro	Ala	Cys	Gln	Val	Thr	Cys	Gln	His	Pro	Glu	Pro	Leu	400
3671	AGC	TGT	GCA	CCT	GCC	TGT	CAA	GTG	ACG	TGT	CAG	CAC	CCT	GAG	CCA	CTG	3718
401	Ala	Cys	Pro	Val	Gln	Cys	Val	Glu	Gly	Cys	His	Ala	His	Cys	Pro	Pro	416
3719	GCC	TGC	CCT	GTG	CAG	TGT	GTG	GAG	GGC	TGC	CAT	GCC	CAT	TGC	CCT	CCA	3766

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Figure 12B (continued)

417	Gly	Lys	Ile	Leu	Asp	Glu	Leu	Gln	Thr	Cys	Val	Asp	Pro	Glu	Asp	432
3767	GGC	AAA	ATC	CTG	GAT	GAG	CTT	TTG	CAG	ACC	TGC	GTT	GAC	CCT	GAA	3814
433	Cys	Pro	Val	Cys	Glu	Val	Ala	Gly	Arg	Arg	Phe	Ala	Ser	Gly	Lys	448
3815	TGT	CCA	GTG	TGT	GAG	GTG	GCT	GGC	CGG	CGT	TTT	GCC	TCA	GCA	AAG	3862
449	Val	Thr	Leu	Asn	Pro	Ser	Asp	Pro	Glu	His	Cys	Gln	Ile	Cys	His	464
3863	GTC	ACC	TTG	AAT	CCC	AGT	GAC	CCT	GAG	CAC	TGC	CAG	ATT	TGC	CAC	3910
465	Asp	Val	Val	Asn	Leu	Thr	Cys	Glu	Ala	Cys	Gln	Glu	Pro	Gly	Gly	480
3911	GAT	GTT	GTC	AAC	CTC	ACC	TGT	GAA	GCC	TGC	CAG	GAG	CCG	GGA	GGC	3958
481	Val	Val	Pro	Pro	Thr	Asp	Ala	Pro	Val	Ser	Pro	Thr	Thr	Leu	Tyr	496
3959	GTG	GTG	CCT	CCC	ACA	GAT	GCC	CCG	GTG	AGC	CCC	ACC	ACT	CTG	TAT	4006
497	Glu	Asp	Ile	Ser	Glu	Pro	Pro	Leu	His	Asp	Phe	Tyr	Cys	Ser	Arg	512
4007	GAG	GAC	ATC	TCG	GAA	CCG	CCG	TTG	CAC	GAT	TTC	TAC	TCC	AGC	AGG	4054
513	Leu	Asp	Leu	Val	Phe	Leu	Leu	Asp	Gly	Ser	Ser	Arg	Leu	Ser	Glu	528
4055	CTG	QAC	CTG	GTC	TTC	CTG	CTG	GAT	GGC	TCC	TCC	AGG	CTG	TCC	GAG	4102

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Figure 12C

529	Glu	Phe	Glu	Val	Leu	Lys	Ala	Phe	Val	Val	Asp	Met	Glu	Arg	Leu	544
4103	GAG	TTT	GAA	GTG	CTG	AAG	GCC	TTT	GTG	GTG	GAC	ATG	ATG	GAG	CGG	CTG
545	Arg	Ile	Ser	Gln	Lys	Trp	Val	Arg	Val	Ala	Val	Val	Glu	Tyr	His	Asp
4151	CGC	ATC	TCC	CAG	AAG	TGG	GTC	CGC	GTG	GCC	GTG	GTG	GAG	TAC	CAC	GAC
561	Gly	Ser	His	Ala	Tyr	Ile	Gly	Leu	Lys	Asp	Arg	Lys	Arg	Pro	Ser	Glu
4199	GGC	TCC	CAC	GCC	TAC	ATC	GGG	CTC	AAG	GAC	CGG	AAG	CGA	CCA	TCA	GAG
577	Leu	Arg	Arg	Ile	Ala	Ser	Gln	Val	Lys	Tyr	Ala	Gly	Ser	Gln	Val	Ala
4247	CTG	CGG	CGC	ATT	GCC	AGC	CAG	GTG	AAG	TAT	GCG	GCG	AGC	CAG	GTG	GCC
593	Ser	Thr	Ser	Glu	Val	Leu	Lys	Tyr	Thr	Leu	Phe	Gln	Ile	Phe	Ser	Lys
4295	TCC	ACC	AGC	AGC	GAG	GTC	TTG	AAA	TAC	ACA	CTG	TTC	CAA	ATC	TTC	AGC
609	Ile	Asp	Arg	Pro	Glu	Ala	Ser	Arg	Ile	Ala	Leu	Leu	Met	Ala	Ser	624
4343	ATC	GAC	CGC	CCT	GAA	GCC	TCC	CGC	ATC	GCC	CTG	CTC	CTG	ATG	GCC	AGC
625	Gln	Glu	Pro	Gln	Arg	Met	Ser	Arg	Asn	Phe	Val	Arg	Tyr	Val	Gln	Gly
4391	CAG	GAG	CCC	CAA	CGG	ATG	TCC	CGG	AAC	TTT	GTC	CGC	TAC	GTC	CAG	GCG
641	Leu	Lys	Lys	Lys	Val	Ile	Val	Ile	Pro	Val	Val	Gly	Ile	Gly	Pro	His
4439	CTG	AAG	AAG	AAG	AAG	GTC	ATT	GTG	ATC	CCG	GTG	GCG	ATT	GGG	CCC	CAT
657	Ala	Asn	Leu	Lys	Gln	Ile	Arg	Leu	Ile	Glu	Lys	Gln	Ala	Pro	Glu	Asn
4487	GCC	AAC	CTC	AAG	CAG	ATC	CGC	CTC	ATC	GAG	AAG	CAG	GCC	CCT	GAG	AAC
673	Lys	Ala	Phe	Val	Leu	Ser	Ser	Val	Asp	Glu	Leu	Glu	Gln	Gln	Arg	Asp
4535	AAG	GCC	TTC	GTG	CTG	AGC	AGT	GTG	GAT	GAG	CTG	GAG	CAG	CAA	AGG	GAC

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Figure 12C (continued)

689	Glu	Ile	Val	Ser	Tyr	Leu	Cys	Asp	Leu	Ala	Pro	Glu	Ala	Pro	Pro	Pro	704
4583	GAG	ATC	GTT	AGC	TAC	CTC	TGT	GAC	CTT	GCC	CCT	GAA	GCC	CCT	CCT	CCT	4630
705	Thr	Leu	Pro	Pro	Asp	Met	Ala	Gln	Val	Thr	Val	Gly	Pro	Gly	Leu	Leu	720
4631	ACT	CTG	CCC	CCC	GAC	ATG	GCA	CAA	GTC	ACT	GTG	GGC	CCG	GGG	CTC	TTG	4678
721	Gly	Val	Ser	Thr	Leu	Gly	Pro	Lys	Arg	Asn	Ser	Met	Val	Leu	Asp	Val	736
4679	GGG	GTT	TCG	ACC	CTG	GGG	CCC	AAG	AGG	AAC	TCC	ATG	GTT	CTG	GAT	GTG	4726
737	Ala	Phe	Val	Leu	Glu	Gly	Ser	Asp	Lys	Ile	Gly	Glu	Ala	Asp	Phe	Asn	752
4727	GCG	TTC	GTC	CTG	GAA	GGA	TCG	GAC	AAA	ATT	GGT	GAA	GCC	GAC	TTC	AAC	4774
753	Arg	Ser	Lys	Glu	Phe	Met	Glu	Glu	Val	Ile	Gln	Arg	Met	Asp	Val	Gly	768
4775	AGG	AGC	AAG	GAG	TTC	ATG	GAG	GAG	GTC	ATT	CAG	CGG	ATG	GAT	GTG	GGC	4822
769	Gln	Asp	Ser	Ile	His	Val	Thr	Val	Leu	Gln	Tyr	Ser	Tyr	Met	Val	Thr	784
4823	CAG	GAC	AGC	ATC	CAC	GTC	ACG	GTG	CTG	CAG	TAC	TCC	TAC	ATG	GTG	ACC	4870
785	Val	Glu	Tyr	Pro	Phe	Ser	Glu	Ala	Gln	Ser	Lys	Gly	Asp	Ile	Leu	Gln	800
4871	GTG	GAG	TAC	CCC	TTC	ACC	GAG	GCA	CAG	TCC	AAA	GGG	GAC	ATC	CTG	CAG	4918

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Figure 12D

801	Arg	Val	Arg	Glu	Ile	Arg	Tyr	Gln	Gly	Gly	Asn	Thr	Thr	Gly	816
4919	CGG	GTG	CGA	GAG	ATC	CGC	TAC	CAG	GGC	GGC	AAC	ACC	ACT	GGG	4966
817	Leu	Ala	Leu	Arg	Tyr	Leu	Ser	Asp	His	Ser	Phe	Leu	Val	Ser	832
4967	CTG	CCC	CTG	CGG	TAC	CTC	TCT	GAC	CAC	ACC	TTC	TTG	GTC	AGC	5014
833	Asp	Arg	Glu	Gln	Ala	Pro	Asn	Leu	Val	Tyr	Met	Val	Thr	Gly	848
5015	GAC	CGG	GAG	CAG	CGG	CCC	AAC	CTG	GTC	TAC	ATG	GTC	ACC	GGA	5062
849	Ala	Ser	Asp	Glu	Ile	Lys	Arg	Leu	Pro	Gly	Asp	Ile	Gln	Val	864
5063	GCC	TCT	GAT	GAG	ATC	AAG	AGG	CTG	CCT	GGA	GAC	ATC	CAG	GTG	5110
865	Ile	Gly	Val	Gly	Pro	Asn	Ala	Asn	Val	Gln	Glu	Leu	Glu	Arg	880
5111	ATT	GGA	GTG	GGC	CCT	AAT	GCC	AAC	GTG	CAG	GAG	CTG	GAG	AGG	5158
881	Trp	Pro	Asn	Ala	Pro	Ile	Leu	Ile	Gln	Asp	Phe	Glu	Thr	Leu	896
5159	TGG	CCC	AAT	GCC	CCT	ATC	CTC	ATC	CAG	GAC	TTT	GAG	ACG	CTC	5206
897	Glu	Ala	Pro	Asp	Leu	Val	Leu	Gln	Arg	Cys	Cys	Ser	Gly	Glu	912
5207	GAG	GCT	CCT	GAC	CTG	GTG	CTG	CAG	AGG	TGC	TGC	TCC	GGA	GAG	5254
913	Gln	Ile	Pro	Thr	Leu	Ser	Pro	Ala	Pro	Asp	Cys	Ser	Gln	Pro	928
5255	CAG	ATC	CCC	ACC	CTC	TCC	CCA	GCA	CCT	GAC	TGC	AGC	CAG	CCC	5302
929	Val	Ile	Leu	Leu	Leu	Asp	Gly	Ser	Ser	Ser	Phe	Pro	Ala	Ser	944
5303	GTG	ATC	CTT	CTC	CTG	GAT	GGC	TCC	TCC	AGT	TTT	CCA	GCT	TCT	5350
945	Asp	Glu	Met	Lys	Ser	Phe	Ala	Lys	Ala	Phe	Ile	Ser	Lys	Ala	960
5351	GAT	GAA	ATG	AAG	AGT	TTC	GCC	AAG	GCT	TTC	ATT	TCA	AAA	GCC	5398

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Figure 12D (continued)

961	Gly	Pro	Arg	Leu	Thr	Gln	Val	Ser	Val	Leu	Gln	Tyr	Gly	Ser	Ile	Thr	976
5399	GGG	CCT	CGT	CTC	ACT	CAG	GTG	TCA	GTG	CTG	CAG	TAT	GGA	AGC	ATC	ACC	5446
977	Thr	Ile	Asp	Val	Pro	Trp	Asn	Val	Val	Pro	Glu	Lys	Ala	His	Leu	Leu	992
5447	ACC	ATT	GAC	GTG	CCA	TGG	AAC	GTG	GTG	CCG	GAG	AAA	GCC	CAT	TTG	CTG	5494
993	Ser	Leu	Val	Asp	Val	Met	Gln	Arg	Glu	Gly	Gly	Pro	Ser	Gln	Ile	Gly	1008
5495	AGC	CTT	GTG	GAC	GTC	ATG	CAG	CGG	GAG	GGA	GCC	CCC	AGC	CAA	ATC	GGG	5542
1009	Asp	Ala	Leu	Gly	Phe	Ala	Val	Arg	Tyr	Leu	Thr	Ser	Glu	Met	His	Gly	1024
5543	GAT	GCC	TTG	GGC	TTT	GCT	GTG	CGA	TAC	TTG	ACT	TCA	GAA	ATG	CAT	GGT	5590
1025	Ala	Arg	Pro	Gly	Ala	Ser	Lys	Ala	Val	Val	Ile	Leu	Val	Thr	Asp	Val	1040
5591	CCC	AGG	CCG	GGA	GCC	TCA	AAG	GCG	GTG	GTC	ATC	CTG	GTC	ACG	GAC	GTC	5638
1041	Ser	Val	Asp	Ser	Val	Asp	Ala	Ala	Ala	Asp	Ala	Ala	Arg	Ser	Asn	Arg	1056
5639	TCT	GTG	GAT	TCA	GTG	GAT	GCA	GCA	GCT	GAT	GCC	GCC	AGG	TCC	AAC	AGA	5686
1057	Val	Thr	Val	Phe	Pro	Ile	Gly	Ile	Gly	Asp	Arg	Tyr	Asp	Ala	Ala	Gln	1072
5687	GTG	ACA	GTG	TTC	CCT	ATT	GGA	ATT	GCA	GAT	CGC	TAC	GAT	GCA	GCC	CAG	5734

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Figure 12E

1073	Leu	Arg	Ile	Leu	Ala	Gly	Pro	Ala	Gly	Asp	Ser	Asn	Val	Val	Lys	Leu	1088
5735	CTA	CGG	ATC	TTG	GCA	GGC	CCA	GCA	GGC	GAC	TCC	AAC	GTG	GTG	AAG	CTC	5782
1089	Gln	Arg	Ile	Glu	Asp	Leu	Pro	Thr	Met	Val	Thr	Leu	Gly	Asn	Ser	Phe	1104
5783	CAG	CGA	ATC	GAA	GAC	CTC	CCT	ACC	ATG	GTC	ACC	TTG	GGC	AAT	TCC	TTC	5830
1105	Leu	His	Lys	Leu	Cys	Ser	Gly	Phe	Val	Arg	Ile	Cys	Met	Asp	Glu	Asp	1120
5831	CTC	CAC	AAA	CTG	TGC	TCT	GGA	TTT	GTT	AGG	ATT	TGC	ATG	GAT	GAG	GAT	5878
1121	Gly	Asn	Glu	Lys	Arg	Pro	Gly	Asp	Val	Trp	Thr	Leu	Pro	Asp	Gln	Cys	1136
5879	GGG	AAT	GAG	AAG	AGG	CCC	GGG	GAC	GTC	TGG	ACC	TTG	CCA	GAC	CAG	TGC	5926
1137	His	Thr	Val	Thr	Cys	Gln	Pro	Asp	Gly	Gln	Thr	Leu	Leu	Lys	Ser	His	1152
5927	CAC	ACC	GTG	ACT	TGC	CAG	CCA	GAT	GGC	CAG	ACC	TTG	CTG	AAG	AGT	CAT	5974
1153	Arg	Val	Asn	Cys	Asp	Arg	Gly	Leu	Arg	Pro	Ser	Cys	Pro	Asn	Ser	Gln	116
5975	CGG	GTC	AAC	TGT	QAC	CGG	GGG	CTG	AGG	CCT	TGC	TGC	CCT	AAC	AGC	CAG	602
1169	Ser	Pro	Val	Lys	Val	Glu	Glu	Thr	Cys	Gly	Cys	Arg	Trp	Thr	Cys	Pro	1184
6023	TCC	CCT	GTT	AAA	GTG	GAA	GAG	ACC	TGT	GGC	TGC	CGC	TGG	ACC	TGC	CCC	6070
1185	Cys	Val	Cys	Thr	Gly	Ser	Ser	Thr	Arg	His	Ile	Val	Thr	Phe	Asp	Gly	1200
6071	TGC	GTG	TGC	ACA	GGC	AGC	TCC	ACT	CGG	CAC	ATC	GTG	ACC	TTT	GAT	GGG	6118
1201	Gln	Asn	Phe	Lys	Leu	Thr	Gly	Ser	Cys	Ser	Tyr	Val	Leu	Phe	Gln	Asn	1216
6119	CAG	AAT	TTC	AAG	CTG	ACT	GGC	AGC	TGT	TCT	TAT	GTC	CTA	TTT	CAA	AAC	6166
1217	Lys	Glu	Gln	Asp	Leu	Glu	Val	Ile	Leu	His	Asn	Gly	Ala	Cys	Ser	Pro	1232
6167	AAG	GAG	CAG	GAC	CTG	GAG	GTG	ATT	CTC	CAT	AAT	GGT	GCC	TGC	AGC	CCT	6214

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Figure 12E (continued)

1233	Gly	Ala	Arg	Gln	Gly	Cys	Met	Lys	Ser	Ile	Glu	Val	Lys	His	Ser	Ala	1248
6215	GGA	GCA	AGG	CAG	GGC	TGC	ATG	AAA	TCC	ATC	GAG	GTG	AAG	CAC	AGT	GCC	6262
1249	Leu	Ser	Val	Glu	Leu	His	Ser	Asp	Met	Glu	Val	Thr	Val	Asn	Gly	Arg	1264
6263	CTC	TCC	GTC	GAG	CTG	CAC	AGT	GAC	ATG	GAG	GTG	ACG	GTG	AAT	GGG	AGA	6310
1265	Leu	Val	Ser	Val	Pro	Tyr	Val	Gly	Gly	Asn	Met	Glu	Val	Asn	Val	Tyr	1280
6311	CTG	GTC	TCT	GTT	CCT	TAC	GTG	GGT	GGG	AAC	ATG	GAA	GTC	AAC	GTT	TAT	6358
1281	Gly	Ala	Ile	Met	His	Glu	Val	Arg	Phe	Asn	His	Leu	Gly	His	Ile	Phe	1296
6359	GGT	CCC	ATC	ATG	CAT	GAG	GTC	AGA	TTC	AAT	CAC	CTT	GGT	CAC	ATC	TTC	6406
1297	Thr	Phe	Thr	Pro	Gln	Asn	Glu	Phe	Phe	Gln	Leu	Gln	Leu	Ser	Pro	Lys	1312
6407	ACA	TTC	ACT	CCA	CAA	AAC	AAT	GAG	TTC	CAA	CTG	CAG	CTC	AGC	CCC	AAG	6454
1313	Thr	Phe	Ala	Ser	Lys	Thr	Tyr	Gly	Leu	Cys	Gly	Ile	Cys	Asp	Glu	Asn	1328
6455	ACT	TTT	GCT	TCA	AAG	ACG	TAT	GGT	CTG	TGT	GGG	ATC	TGT	GAT	GAG	AAC	6502
1329	Gly	Ala	Asn	Asp	Phe	Met	Leu	Arg	Asp	Gly	Thr	Val	Thr	Thr	Asp	Trp	1344
6503	GGA	CCC	AAT	GAC	TTC	ATG	CTG	AGG	GAT	GGC	ACA	GTC	ACC	ACA	GAC	TGG	6550

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Figure 12F

1345	Lys	Thr	Leu	Val	Gln	Glu	Trp	Thr	Val	Gln	Arg	Pro	Gly	Gln	Thr	Cys	1360
6551	AAA	ACA	CTT	GTT	CAG	GAA	TGG	ACT	GTG	CAG	CGG	CCA	GGA	CAG	ACG	TGC	6598
1361	Gln	Pro	Ile	Leu	Glu	Glu	Gln	Cys	Leu	Val	Pro	Asp	Ser	Ser	His	Cys	1376
6599	CAG	CCC	ATC	CTG	CAG	GAG	CAG	TGT	CTT	GTC	CCC	GAC	AGC	TCC	CAC	TGC	6646
1377	Gln	Val	Leu	Leu	Leu	Pro	Leu	Phe	Ala	Glu	Cys	His	Lys	Val	Leu	Ala	1392
6647	CAG	GTC	CTC	CTC	TTA	CCA	CTG	TTT	GCT	GAA	TGC	CAC	AAG	GTC	CTG	GCT	6694
1393	Pro	Ala	Thr	Phe	Tyr	Ala	Ile	Cys	Gln	Gln	Asp	Ser	Ser	His	Gln	Glu	1408
6695	CCA	GCC	ACA	TTC	TAT	GCC	ATC	TGC	CAG	CAG	GAC	AGT	TCG	CAC	CAG	GAG	6742
1409	Gln	Val	Cys	Glu	Val	Ile	Ala	Ser	Tyr	Ala	His	Leu	Cys	Arg	Thr	Asn	1424
6743	CAA	GTG	TGT	GAG	GTG	ATC	GCC	TCT	TAT	GCC	CAC	CTC	TGT	CGG	ACC	AAC	6790
1425	Gly	Val	Cys	Val	Asp	Trp	Arg	Thr	Pro	Asp	Phe	Cys	Ala	Met	Ser	Cys	1440
6791	GGG	GTC	TGC	GTT	GAC	TGG	AGG	ACA	CCT	GAT	TTC	TGT	GCT	ATG	TCA	TGC	6838
1441	Pro	Pro	Ser	Leu	Val	Tyr	Asn	His	Cys	Glu	His	Gly	Cys	Pro	Arg	His	1456
6839	CCA	CCA	TCT	CTG	GTC	TAC	AAC	CAC	TGT	GAG	CAT	GGC	TGT	CCC	CGG	CAC	6886
1457	Cys	Asp	Gly	Asn	Val	Ser	Ser	Cys	Gly	Asp	His	Pro	Ser	Glu	Gly	Cys	1472
6887	TGT	GAT	GGC	AAC	GTG	AGC	TCC	TGT	GGG	GAC	CAT	CCC	TCC	GAA	GGC	TGT	6934
1473	Phe	Cys	Pro	Pro	Asp	Lys	Val	Met	Leu	Glu	Gly	Ser	Cys	Val	Pro	Glu	1488
6935	TTC	TGC	CCT	CCA	GAT	AAA	GTG	ATG	TTG	GAA	GGC	AGC	TGT	GTC	CCT	GAA	6982
1489	Glu	Ala	Cys	Thr	Gln	Cys	Ile	Gly	Glu	Asp	Gly	Val	Gln	His	Gln	Phe	1504
6983	GAG	GCC	TGC	ACT	CAG	TGC	ATT	GGT	GAG	GAT	GGA	GTC	CAG	CAC	CAG	TTC	7030

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Figure 12F (continued)

1505	Leu	Glu	Ala	Trp	Val	Pro	Asp	His	Gln	Pro	Cys	Gln	Ile	Cys	Thr	Cys	1520
7031	CTG	GAA	GCC	TGG	GTC	CCG	GAC	CAC	CAG	CCC	TGT	CAG	ATC	TGC	ACA	TGC	7078
1521	Leu	Ser	Gly	Arg	Lys	Val	Asn	Cys	Thr	Thr	Gln	Pro	Cys	Pro	Thr	Ala	1536
7079	CTC	AGC	GGG	CGG	AAG	GTC	AAC	TGC	ACA	ACG	CAG	CCC	TGC	CCC	ACG	GCC	7126
1537	Lys	Ala	Pro	Thr	Cys	Gly	Leu	Cys	Glu	Val	Ala	Arg	Leu	Arg	Gln	Asn	1552
7127	AAA	GCT	CCC	ACG	TGT	GGC	CTG	TGT	GAA	GTA	GCC	CGC	CTC	CGC	CAG	AAT	7174
1553	Ala	Asp	Gln	Cys	Cys	Pro	Glu	Tyr	Glu	Cys	Val	Cys	Asp	Pro	Val	Ser	1568
7175	GCA	GAC	CAG	TGC	TGC	CCC	GAG	TAT	GAG	TGT	GTG	TGT	GAC	CCA	GTG	AGC	7222
1569	Cys	Asp	Leu	Pro	Pro	Val	Pro	His	Cys	Glu	Arg	Gly	Leu	Gln	Pro	Thr	1584
7223	TGT	GAC	CTG	CCC	CCA	GTG	CCT	CAC	TGT	GAA	CGT	GGC	CTC	CAG	CCC	ACA	7270
1585	Leu	Thr	Asn	Pro	Gly	Glu	Cys	Arg	Pro	Asn	Phe	Thr	Cys	Ala	Cys	Arg	1600
7271	CTG	ACC	AAC	CCT	GGC	GAG	TGC	AGA	CCC	AAC	TTC	ACC	TGC	GCC	TGC	ACG	7318
1601	Lys	Glu	Glu	Cys	Lys	Arg	Val	Ser	Pro	Pro	Ser	Cys	Pro	Pro	His	Arg	1616
7319	AAG	GAG	GAG	TGC	AAA	AGA	GTG	TCC	CCA	CCC	TCC	TGC	CCC	CCG	CAC	CCT	7366

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Figure 12G

1617	Leu	Pro	Thr	Leu	Arg	Lys	Thr	Gln	Cys	Cys	Asp	Glu	Tyr	Glu	Cys	Ala	1632
7367	TTG	CCC	ACC	CTT	CGG	AAG	ACC	CAG	TGC	TGT	GAT	GAG	TAT	GAG	TGT	GCC	7414
1633	Cys	Asn	Cys	Val	Asn	Ser	Thr	Val	Ser	Cys	Pro	Leu	Gly	Tyr	Leu	Ala	1648
7415	TGC	AAC	TGT	GTC	AAC	TCC	ACA	GTG	AGC	TGT	CCC	CTT	GGG	TAC	TTG	GCC	7462
1649	Ser	Thr	Ala	Thr	Asn	Asp	Cys	Gly	Cys	Thr	Thr	Thr	Thr	Cys	Leu	Pro	1664
7463	TCA	ACC	GCC	ACC	AAT	GAC	TGT	GCC	TGT	ACC	ACA	ACC	ACC	TGC	CTT	CCC	7510
1665	Asp	Lys	Val	Cys	Val	His	Arg	Ser	Thr	Ile	Tyr	Pro	Val	Gly	Gln	Phe	1680
7511	GAC	AAG	GTG	TGT	GTC	CAC	CGA	AGC	ACC	ATC	TAC	CCT	GTG	GGC	CAG	TTC	7558
1681	Trp	Glu	Glu	Gly	Cys	Asp	Val	Cys	Thr	Cys	Thr	Asp	Met	Glu	Asp	Ala	1696
7559	TGG	GAG	GAG	GGC	TGC	GAT	GTG	TGC	ACC	TGC	ACC	GAC	ATG	GAG	GAT	GCC	7606
1697	Val	Met	Gly	Leu	Arg	Val	Ala	Gln	Cys	Ser	Gln	Lys	Pro	Cys	Glu	Asp	1712
7607	GTG	ATG	GGC	CTC	CGC	GTG	GCC	CAG	TGC	TCC	CAG	AAG	CCC	TGT	GAG	GAC	7654
1713	Ser	Cys	Arg	Ser	Gly	Phe	Thr	Tyr	Val	Leu	His	Glu	Gly	Glu	Cys	Cys	1728
7655	ACC	TGT	CGG	TCG	GGC	TTC	ACT	TAC	GTT	CTG	CAT	GAA	GGC	GAG	TGC	TGT	7702
1729	Gly	Arg	Cys	Leu	Pro	Ser	Ala	Cys	Glu	Val	Val	Thr	Gly	Ser	Pro	Arg	1744
7703	GGG	AGG	TGC	CTG	CCA	TCT	GCC	TGT	GAG	GTG	GTG	ACT	GGC	TCA	CCG	CGG	7750
1745	Gly	Asp	Ser	Gln	Ser	Ser	Trp	Lys	Ser	Val	Gly	Ser	Gln	Trp	Ala	Ser	1760
7751	GGG	GAC	TCC	CAG	TCT	TCC	TGG	AAG	AGT	GTC	GGC	TCC	CAG	TGG	GCC	TCC	7798
1761	Pro	Glu	Asn	Pro	Cys	Leu	Ile	Asn	Glu	Cys	Val	Arg	Val	Lys	Glu	Glu	1776
7799	CCG	GAG	AAC	CCC	TGC	CTC	ATC	AAT	GAG	TGT	GTC	CGA	GTG	AAG	GAG	GAG	7846

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Figure 12G (continued)

1777	Val	Phe	Ile	Gln	Gln	Arg	Asn	Val	Ser	Cys	Pro	Gln	Leu	Glu	Val	Pro	1792
7847	GTC	TTT	ATA	CAA	CAA	AGG	AAC	GTC	TCC	TGC	CCC	CAG	CTG	GAG	GTC	CCT	7894
1793	Val	Cys	Pro	Ser	Gly	Phe	Gln	Leu	Ser	Cys	Lys	Thr	Ser	Ala	Cys	Cys	1808
7895	GTC	TGC	CCC	TCG	GGC	TTT	CAG	CTG	AGC	TGT	AAG	ACC	TCA	GCG	TGC	TGC	7942
1809	Pro	Ser	Cys	Arg	Cys	Glu	Arg	Met	Glu	Ala	Cys	Met	Leu	Asn	Gly	Thr	1824
7943	CCA	AGC	TGT	CGC	TGT	GAG	CGC	ATG	GAG	GCC	TGC	ATG	CTC	AAT	GGC	ACT	7990
1825	Val	Ile	Gly	Pro	Gly	Lys	Thr	Val	Met	Ile	Asp	Val	Cys	Thr	Thr	Cys	1840
7991	GTC	ATT	GGG	CCC	GGG	AAG	ACT	GTG	ATG	ATC	GAT	GTG	TGC	ACG	ACC	TGC	8038
1841	Arg	Cys	Met	Val	Gln	Val	Gly	Val	Ile	Ser	Gly	Phe	Lys	Leu	Glu	Cys	1856
8039	CGC	TGC	ATG	GTG	CAG	GTG	GGG	GTC	ATC	TCT	GGA	TTC	AAG	CTG	GAG	TGC	8086
1857	Arg	Lys	Thr	Thr	Cys	Asn	Pro	Cys	Pro	Leu	Gly	Tyr	Lys	Glu	Glu	Asn	1872
8087	AGG	AAG	ACC	ACC	TGC	AAC	CCC	TGC	CCC	CTG	GGT	TAC	AAG	GAA	GAA	AAT	8134
1873	Asn	Thr	Gly	Glu	Cys	Cys	Gly	Arg	Cys	Leu	Pro	Thr	Ala	Cys	Thr	Ile	1888
8135	AAC	ACA	GGT	GAA	TGT	TGT	GGG	ACA	TGT	TTG	CCT	ACG	GCT	TGC	ACC	ATT	8182

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Figure 12H

1889	Gln	Leu	Arg	Gly	Gly	Gln	Ile	Met	Thr	Leu	Lys	Arg	Asp	Glu	Thr	Leu	1904
8183	CAG	CTA	AGA	GGA	GGA	CAG	ATC	ATG	ACA	CTG	AAG	CGT	GAT	GAG	ACG	CTC	8230
1905	Gln	Asp	Gly	Cys	Asp	Thr	His	Phe	Cys	Lys	Val	Asn	Glu	Arg	Gly	Glu	1920
8231	CAG	GAT	GGC	TGT	GAT	ACT	CAC	TTC	TGC	AAG	GTC	AAT	GAG	AGA	GGA	GAG	8278
1921	Tyr	Phe	Trp	Glu	Lys	Arg	Val	Thr	Gly	Cys	Pro	Pro	Phe	Asp	Glu	His	1936
8279	TAC	TTC	TGG	GAG	AAG	AGG	GTC	ACA	GGC	TGC	CCA	CCC	TTT	GAT	GAA	CAC	8326
1937	Lys	Cys	Leu	Ala	Glu	Gly	Gly	Lys	Ile	Met	Lys	Ile	Pro	Gly	Thr	Cys	1952
8327	AAG	TGT	CTG	GCT	GAG	GGA	GCT	AAA	ATT	ATG	AAA	ATT	CCA	GGC	ACC	TGC	8374
1953	Cys	Asp	Thr	Cys	Glu	Glu	Pro	Glu	Cys	Asn	Asp	Ile	Thr	Ala	Arg	Leu	1968
8375	TGT	GAC	ACA	TGT	GAG	GAG	CCT	GAG	TGC	AAC	GAC	ATC	ACT	GCC	AGG	CTG	8422
1969	Gln	Tyr	Val	Lys	Val	Gly	Ser	Cys	Lys	Ser	Glu	Val	Glu	Val	Asp	Ile	1984
8423	CAG	TAT	GTC	AAG	GTG	GGA	AGC	TGT	AAG	TCT	GAA	GTA	GAG	GTG	GAT	ATC	8470
1985	His	Tyr	Cys	Gln	Gly	Lys	Cys	Ala	Ser	Lys	Ala	Met	Tyr	Ser	Ile	Asp	2000
8471	CAC	TAC	TGC	CAG	GGC	AAA	TGT	GCC	AGC	AAA	GCC	ATG	TAC	TCC	ATT	GAC	8518
2001	Ile	Asn	Asp	Val	Gln	Asp	Gln	Cys	Ser	Cys	Cys	Ser	Pro	Thr	Arg	Thr	2016
8519	ATC	AAC	GAT	GTG	CAG	GAC	CAG	TGC	TCC	TGC	TGC	TCT	CCG	ACA	CGG	ACG	8566
2017	Glu	Pro	Met	Gln	Val	Ala	Leu	His	Cys	Thr	Asn	Gly	Ser	Val	Val	Tyr	2032
8567	GAG	CCC	ATG	CAG	GTG	GCC	CTG	CAC	TGC	ACC	AAT	CGC	TCT	GTT	GTG	TAC	8614
2033	His	Glu	Val	Leu	Asn	Ala	Met	Glu	Cys	Lys	Cys	Ser	Pro	Arg	Lys	Cys	2048
8615	CAT	GAG	GTT	CTC	AAT	GCC	ATG	GAG	TGC	AAA	TGC	TCC	CCC	AGG	AAG	TGC	8662
2049	Ser	Lys	***														
8663	AGC	AAG	TGA														

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Figure 13

1	Met	Leu	His	Asp	Phe	Tyr	Cys	Ser	Arg	Leu	Leu	Asp	Leu	Val	Phe	Leu	16
1	ATG	TTG	CAC	GAT	TTC	TAC	TGC	AGC	AGG	CTA	CTG	GAC	CTG	GTC	TTC	CTG	48
17	Leu	Asp	Gly	Ser	Ser	Arg	Leu	Ser	Glu	Ala	Glu	Phe	Glu	Val	Leu	Lys	32
49	CTG	GAT	GGC	TCC	TCC	AGG	CTG	TCC	GAG	GCT	GAG	TTT	GAA	GTG	CTG	AAG	96
33	Ala	Phe	Val	Val	Asp	Met	Met	Glu	Arg	Leu	Arg	Ile	Ser	Gln	Lys	Trp	48
97	GCC	TTT	GTG	GTG	GAC	ATG	ATG	GAG	CGG	CTG	CGC	ATC	TCC	CAG	AAG	TGG	144
49	Val	Arg	Val	Ala	Val	Val	Glu	Tyr	His	Asp	Gly	Ser	His	Ala	Tyr	Ile	64
145	GTC	CGC	GTG	GCC	GTG	GTG	GAG	TAC	CAC	GAC	GGC	TCC	CAC	GCC	TAC	ATC	192
65	Gly	Leu	Lys	Asp	Arg	Lys	Arg	Pro	Ser	Glu	Leu	Arg	Arg	Ile	Ala	Ser	80
193	GGG	CTC	AAG	GAC	GAC	CGG	AAG	CGA	CCA	TCA	GAG	CTG	CGG	CGC	ATT	GCC	240
81	Gln	Val	Lys	Tyr	Ala	Gly	Ser	Gln	Val	Ala	Ser	Thr	Ser	Glu	Val	Leu	96
241	CAG	GTG	AAG	TAT	GCG	GCG	AGC	AGC	CAG	GTG	GCC	TCC	ACC	AGC	GAG	TTG	288
97	Lys	Tyr	Thr	Leu	Phe	Gln	Ile	Phe	Ser	Lys	Ile	Asp	Arg	Pro	Glu	Ala	112
289	AAA	TAC	ACA	CTG	TTC	CAA	ATC	TTC	AGC	AAG	ATC	GAC	CGC	CCT	GAA	GCC	336
113	Ser	Arg	Ile	Ala	Leu	Leu	Leu	Met	Ala	Ser	Gln	Glu	Pro	Gln	Arg	Met	128
337	TCC	CGC	ATC	GCC	CTG	CTC	CTG	ATG	GCC	AGC	CAG	GAG	CCC	CAA	CGG	ATG	384
129	Ser	Arg	Asn	Phe	Val	Arg	Tyr	Val	Gln	Gly	Leu	Lys	Lys	Lys	Lys	Val	144
385	TCC	CGG	AAC	TTT	GTC	CGC	TAC	GTC	CAG	GGC	CTG	AAG	AAG	AAG	AAG	GTC	432
145	Ile	Val	Ile	Pro	Val	Gly	Ile	Gly	Pro	His	Ala	Asn	Leu	Lys	Gln	Ile	160
433	ATT	GTG	ATC	CCG	GTG	GGC	ATT	GGG	CCC	CAT	GCC	AAC	CTC	AAG	CAG	ATC	480

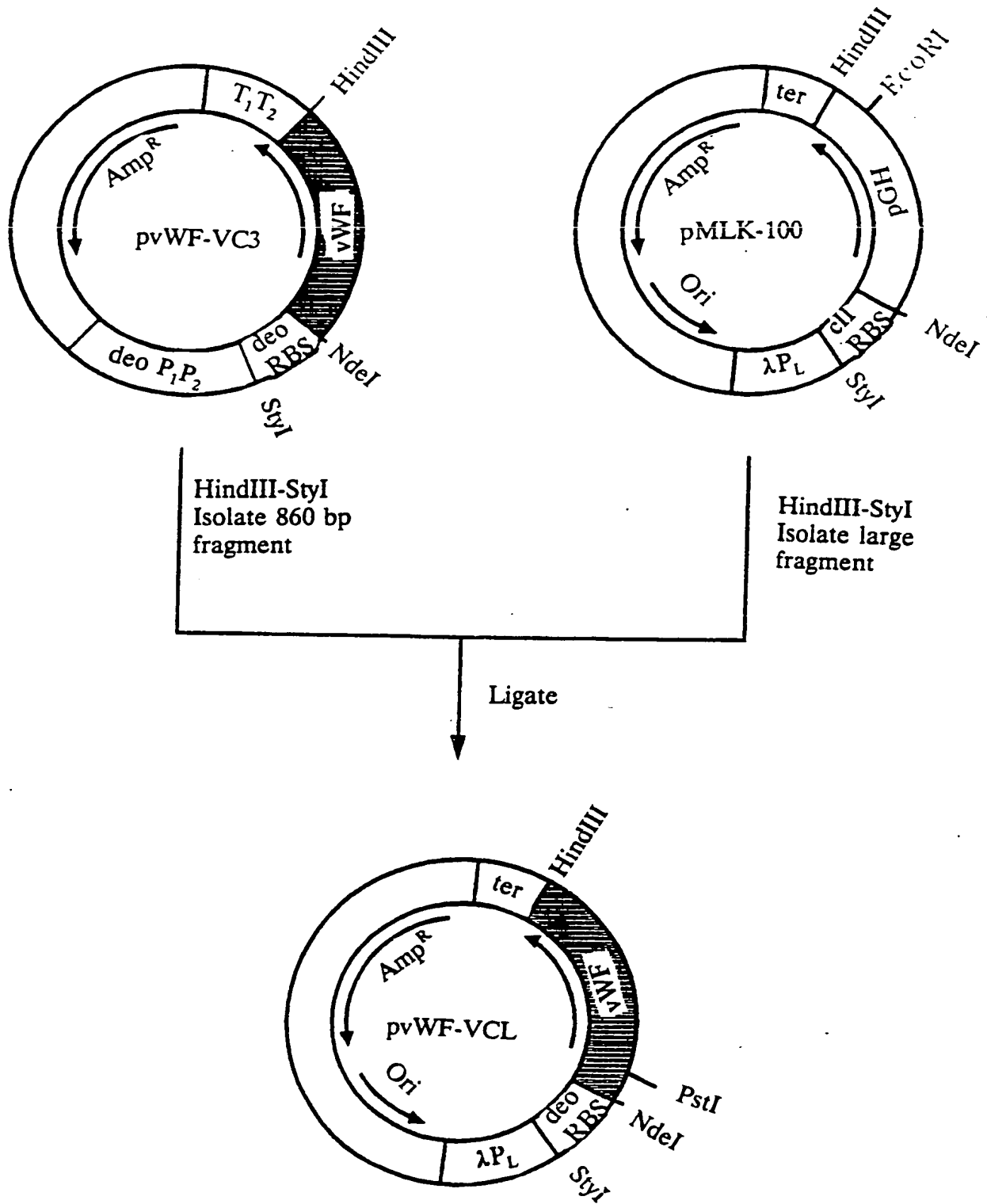
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Figure 13 (continued)

161	Arg	Leu	Ile	Glu	Lys	Gln	Ala	Pro	Glu	Asn	Lys	Ala	Phe	Val	Leu	Ser	176
481	CGC	CTC	ATC	GAG	AAG	CAG	GCC	CCT	GAG	AAC	AAG	GCC	TTC	GTG	CTG	ACC	528
177	Ser	Val	Asp	Glu	Leu	Glu	Gln	Gln	Arg	Asp	Glu	Ile	Val	Ser	Tyr	Leu	192
529	AGT	GTG	GAT	GAG	CTG	GAG	CAG	CAA	AGG	GAC	GAG	ATC	GTT	AGC	TAC	CTC	576
193	Cys	Asp	Leu	Ala	Pro	Glu	Ala	Pro	Pro	Pro	Thr	Leu	Pro	Pro	Asp	Met	208
577	TGT	GAC	CTT	GCC	CCT	GAA	GCC	CCT	CCT	CCT	ACT	CTG	CCC	CCC	GAC	ATG	624
209	Ala	Gln	Val	Thr	Val	Gly	Pro	Gly	Leu	Leu	Gly	Val	Ser	Thr	Leu	Gly	224
625	GCA	CAA	GTC	ACT	GTG	GGC	CCG	GGG	CTC	TTG	GGG	GTT	TCG	ACC	CTG	GGG	672
225	Pro	Lys	***														
673	CCC	AAG	TAA														

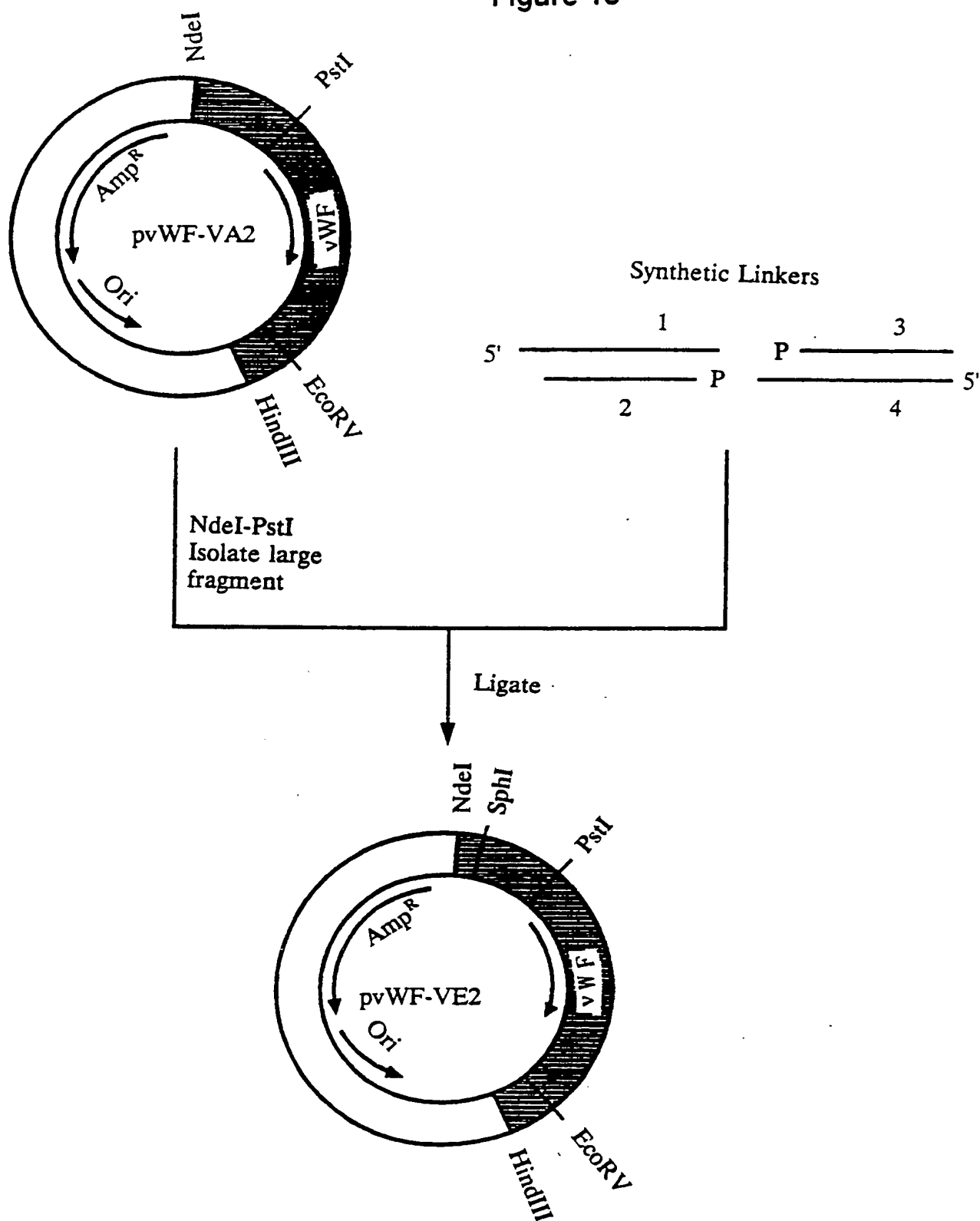
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Figure 14



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Figure 15



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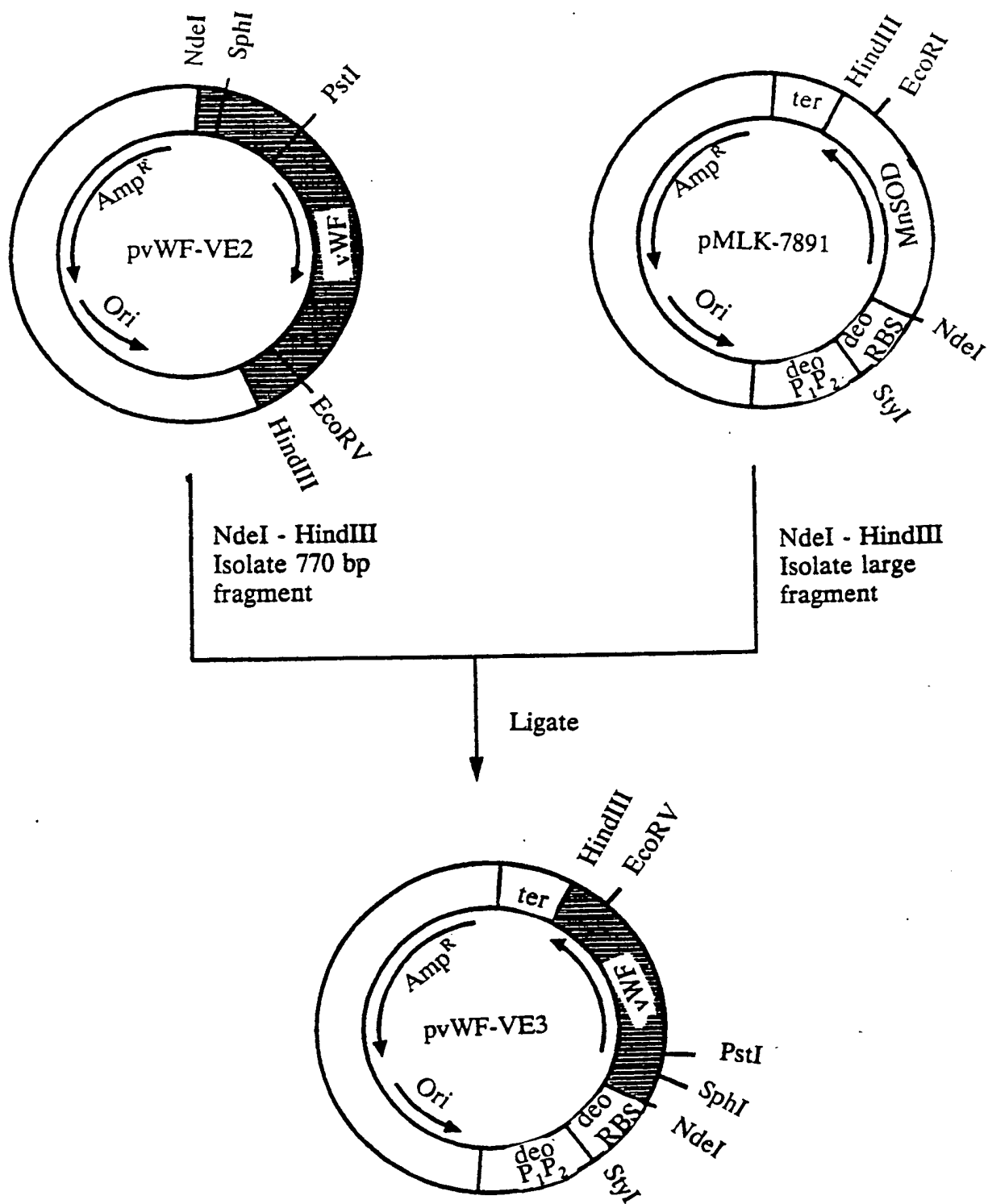
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Figure 16

<u>Linker</u>	
No. 1	5' TATGCTCACCCTGTGAAGCATGCCAGGAGCCGGAGGCCCTGGTGGCTCCACAGATGCCCCCGGTG
No. 2	3' ACGAGTGGACACTTTCGTACGGTCTCTCGGCCCTCCGGACCCACCACGGAGGGTGTCTACGGGG
No. 3	5' AGCCCCACCACCTCTGTATGTGGAGGACATCTCGGAACCGCCGTTGCACGATTTCTACTGCA
No. 4	3' CCACTCGGGGTGGTGAGACATACACCTCCTGTAGAGCCTTGGCGGCAACGTGCTAAAGATG

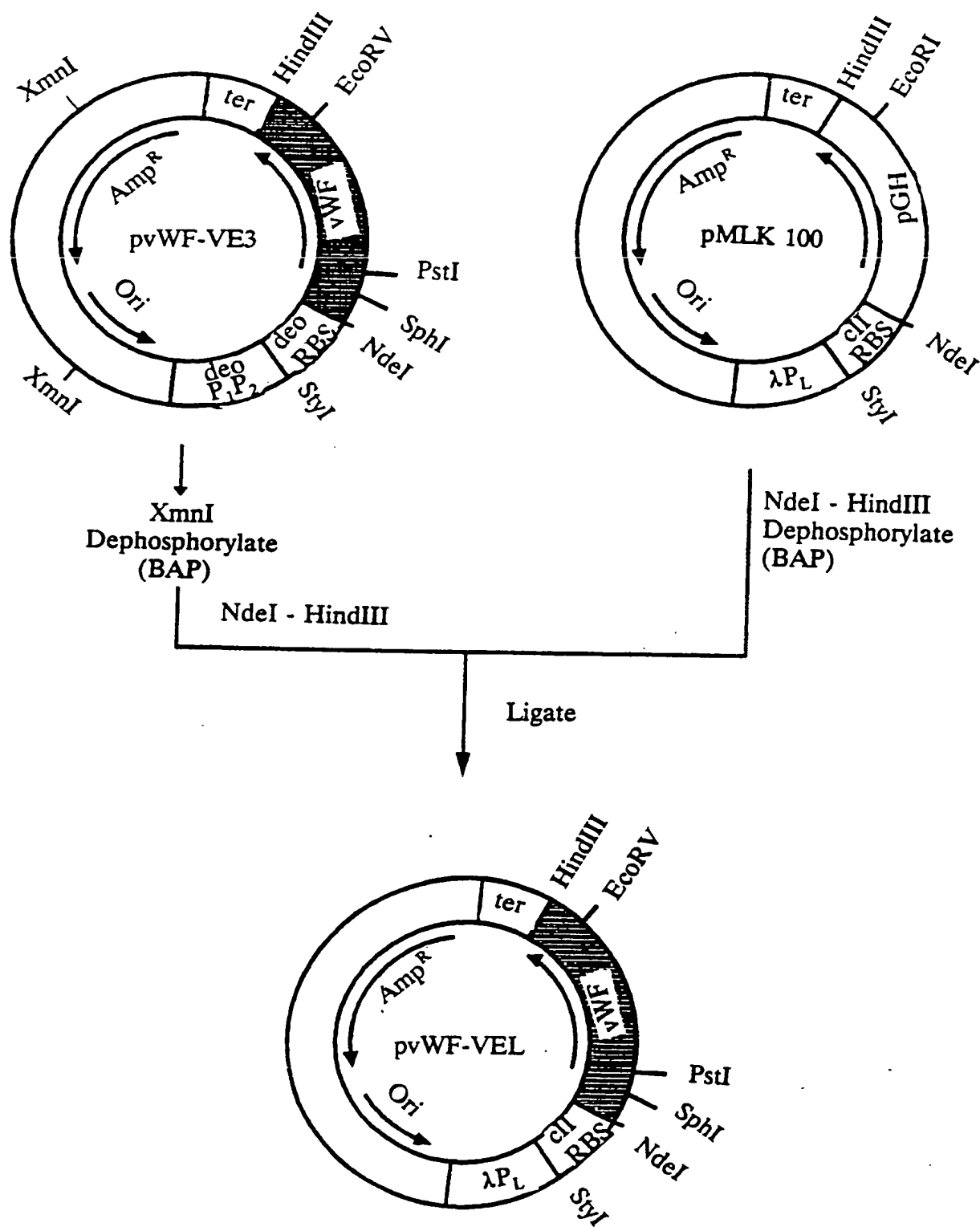
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Figure 17



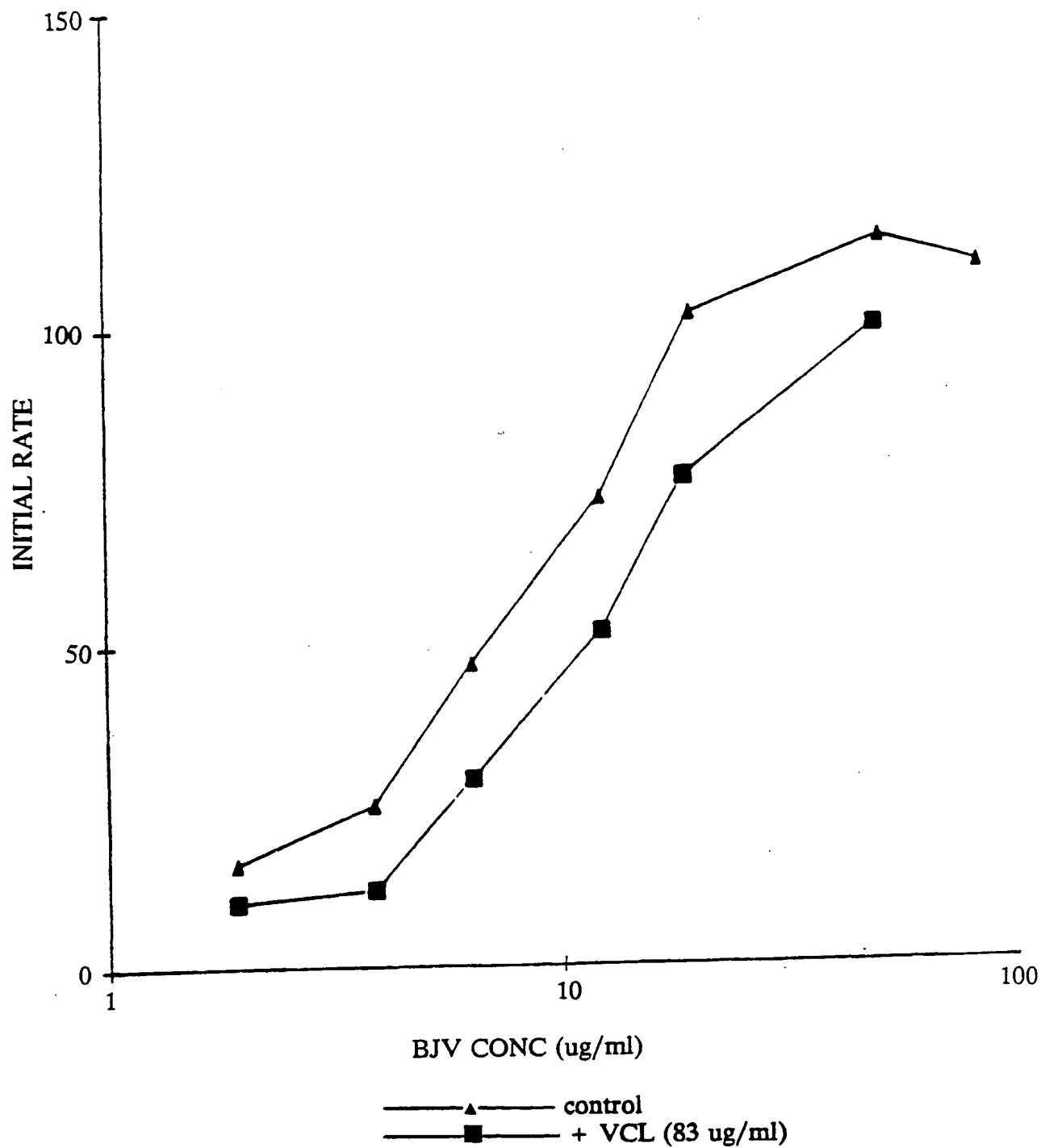
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Figure 18



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Figure 19

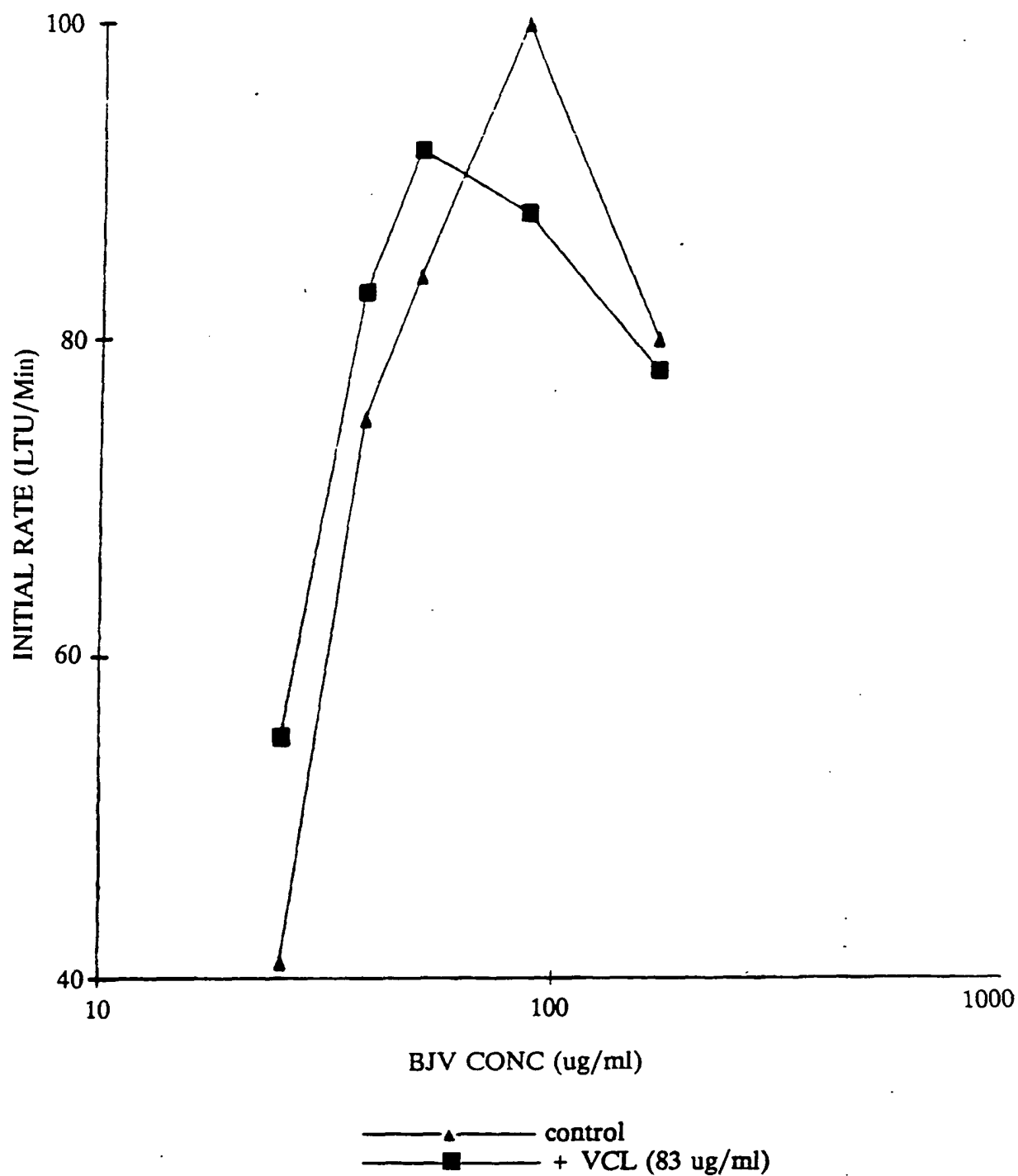


LTU = Light transmission units

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Figure 20



LTU = Light transmission units

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01416

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07K 13/00; A61K 37/00; C12N 1/21, 15/14, 15/70 US, CL.: 530/381, 383; 514/12; 435/69.6, 252.3, 320.1		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
US.CL.	530/381, 383; 514/12; 435/69.6, 252.3, 320.1	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Databases: Automated Patent Searching (1975-1991) Dialog (Files: World Patents Index, CAS, Biosis, Medline)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Biochemical and Biophysical Research Communications, Volume 164, No. 3, issued 15 November 1989, Peitu et al., "Production in Escherichia Coli of a Biologically Active Subfragment of Von Willebrand Factor Corresponding to the Platelet Glycoprotein Ib, Collagen and Heparin Binding Domains," pages 1339-1347, see entire document.	1-11,16
Y	The Journal of Biological Chemistry, Volume 263, No. 34, issued 05 December 1988, Mohri et al., "Structure of the Von Willebrand Factor Domain Interacting with Glycoprotein Ibs" pages 17901-17904, see entire document.	1-11,16
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
30 May 1991		<div style="font-size: 1.2em; font-weight: bold;">24 JUN 1991</div>
International Searching Authority		Signature of Authorized Officer
ISA/US		<div style="text-align: center;"> R. Keith Baker, Ph.D. </div>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Biological Chemistry. Volume 261, No. 32, issued 15 November 1986. Pareti et al., "Isolation and Characterization of a Collagen Binding Domain in Human Von Willebrand Factor", pages 15310-15315, see the Abstract.	1-11.16
Y	The Journal of Biological Chemistry, Volume 262, No. 4 issued 05 February 1987, Fujimura et al., "A Heparin- binding Domain of Human von Willebrand Factor" pages 1734-1739, see the entire document.	1-11.16

FURTHER INFORMATION

CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-11 and 16

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT/ISA/210

GROUP I. Claims 1-11 and 16 are drawn to a first product, a first method of making the product and a first method of using the product comprising the non-glycosylated polypeptide, a recombinant method of expression and a method of treating platelet aggregation, classified in Classes 530, 435 and 514, subclasses 381, 69.6 and 12. Group II, claim 12 is drawn to a second method of use comprising treatment of cerebrovascular disorders, classified in Class 514, subclass 12. Group III, claims 13-15 is drawn to a third method of use comprising treatment of cardiovascular disorders, classified in Class 514, subclass 12. Group IV, claim 17 is drawn to fourth method of use comprising treatment for blood vessel patency, classified in Class 514, subclass 12. Group V, claim 18, is drawn to a fifth method of use comprising treatment of cancer, classified in Class 514, subclass 12. Group VI, claims 19, 26 and 30, is drawn to a sixth method of use comprising treatment for thrombus inhibition, classified in Class 514, subclass 12. Group VII, claims 21-22, is drawn to a seventh method of use comprising treatment of platelet adhesion, classified in Class 514, subclass 12. Group VIII, claim 23 is drawn to a eighth method of use comprising treatment of re-occlusion, classified in Class 514, subclass 12. Group IX, claim 24 is drawn to a ninth method of use comprising treatment of vaso-occlusive crises, classified in Class 514, subclass 12. Group X, claim 25 is drawn to a tenth method of use comprising treatment of arteriosclerosis, classified in Class 514, subclass 12. Group XI, claims 27-28, is drawn to an eleventh method of use comprising treatment of platelets

activation, classified in Class 514, subclass 12. Group XII, claim 29, is drawn to a twelvth method of use comprising treatment of stenosis, classified in Class 514, subclass 12. Group XIII, claims 31-38, is drawn to a second method of making the first product, classified in class 530, subclass 381 for example. Group XIV, claim 20, is drawn to a second product comprising the polypeptide bound to a solid matrix classified in class 530, subclass 381.

PCT Rule 13.2 permits claims to "a" (one) product and "a" (one) method of making and "a" (one) method of using said product. The invention of Group I constitutes a combination of a first product, a first method of making the product and a first method of use. Groups II-XII are drawn to different in vivo methods of use of the first product in the treatment of distinct disease states. Group XIII is drawn to a second and distinct method of making the first product comprising different method steps from those in Group I. Group XIV is drawn to a second and distinct product from the first product of Group I.